REMARKS

The applicant provides the following remarks which Applicant believes addresses each concern raised in the non-final action mailed October 21, 2009.

1. Status Of The Claims.

Claims 1, 4-12, 15, 16, 18, 21, 150, 151, 154, and 155 are pending in the subject application.

Claims 1, 4-12, 15, 16, 18, 21, 151, 154 and 155 are rejected under 35 U.S.C.A Section 103(a) as being unpatentable over the combination of each of WO 02/41906 ("WO '906") and WO 01/37655 (WO '655) in view of each of Tardif et al. ("Tardiff") and United States Patent No. 6,140,121 to Ellington ("Ellington") supported by Padilla et al ("Padilla"), Johnson ("Johnson"), and Seidel et al ("Seidel").

Claims 1, 4-12, 15, 16, 18, 21, 148, 149, 150, and 152-154 are rejected under 35 U.S.C.A Section 103(a) as being unpatentable over the combination of each of WO 02/41906 and WO 01/37655 in view of Tardif et al. ("Tardiff") and further in view of each of WO 02/28311 and Lindsey et al ("Lindsey") supported by Johnson and Seidel.

Claims 2-4, 13-14, 17, 9-20, 22-149, and 152-153 have been without prejudice canceled. Claim 1 has been amended without the addition of any new matter. Applicant respectfully reserves the right to pursue any non-elected claims, canceled or otherwise unclaimed subject matter in one or more continuation, continuation-in-part, or divisional applications. Claims 5, 15, 18, 150, 151, 154, and 155 have been previously presented. Claims 6, 7, 8, 9, 11, 12, and 21 are as originally presented.

2. The Seidel Reference Must Be Withdrawn Because Applicant's Priority Date Falls Before the Publication Date Of The Seidel Reference.

Applicant submits evidence that the reference entitled "Current Status of Sexing Mammalian Spermatozoa" by Seidel et al. was published on December 1, 2002. *See attached Exhibit A.* Applicant's priority date based on United States 60/400,486 is July 22, 2002. Accordingly, Applicant respectfully requests that the Examiner withdraw the Seidel reference.

3. Applicant Is A Co-Author Or Can Swear Behind The Lindsey Reference.

The Lindsey reference was published on December 3, 2001. *See attached Exhibit B.* Applicant's priority date based on United States 60/400,486 is July 22, 2002. The Lindsey reference was published less than one year before the priority date of the instant application. Lindsey is a named inventor and a co-author of the Lindsey reference. Accordingly, Applicant reserves the right to overcome the rejection based the Lindsey reference by either swearing behind the reference by way of a Section 1.131 Declaration or by way of a Section 1.132 Declaration establishing that the article is describing Applicant's own work.

4. The Rejections Under 35 U.S.C.A. Section 103 (a) Are Overcome.

The Examiner has rejected claims 1, 4-12, 15, 16, 18, 21, 151, 154 and 155 under 35 U.S.C.A Section 103(a) as being unpatentable over the combination of each of WO 02/41906 and WO 01/37655 in view of each of Tardif et al. ("Tardiff") and United States Patent No. 6,140,121 to Ellington ("Ellington") supported by Padilla et al ("Padilla"), Johnson ("Johnson"), and Seidel et al ("Seidel").

The Examiner has also rejected claims 1, 4-12, 15, 16, 18, 21, 148, 149, 150, and 152-154 under 35 U.S.C.A Section 103(a) as being unpatentable over the combination of each of WO 02/41906 and WO 01/37655 in view of Tardif et al. ("Tardiff") and further in view of each of WO 02/28311 and Lindsey et al ("Lindsey") supported by Johnson and Seidel.

To reject a claim based on combining prior art elements according to known methods, the office must resolve the Graham factual inquires and provide a finding that the prior art included each element claimed. *MPEP §2143 A (1).* The rationale to support a conclusion that the claim would have been obvious is that all the claimed elements were known in the prior art and one skilled in the art could have combined the elements as claimed by known methods with no change in their respective functions, and the combination yielded nothing more than predictable results to one of ordinary skill in the art. *KSR, 550 US at ___ 82, USPQ2d at 1395.* If any these findings cannot be made, then this rationale cannot be used to support a conclusion that the claim would have been obvious to one of ordinary skill in the art. *MPEP §2143 A (1).* Additionally, "If the proposed modification or combination of the prior art would change the principal of operation of the prior art invention being modified, then the teaching of the references are not sufficient to render claims prima facie obvious" MPEP Rule 2143.01 VI.

The combination of either WO'655 or WO'906 in view of each of Tardif et al. ("Tardiff") and United States Patent No. 6,140,121 to Ellington ("Ellington").

Not All Limitations of the Claimed Invention Are Taught.

To reject a claim based on combining prior art elements according to known methods, the office must resolve the Graham factual inquires and provide a finding that the prior art included each element claimed. *MPEP §2143 A (1).*

The Examiner indicates that the WO'655 reference teaches that "incubating the semen sample at temperatures ranging from 5-25°C (p.10, lines 10-25). *Office Action at Page 4.* However, the cited teaching refers to cooling sperm cells that have already been sex selected by flow cytometry. *See WO '655, page 10, lines 12-13.* Applicant claims "incubating said semen at a temperature above which sperm cell membrane lipids transition from a liquid phase to gel phase". Applicant has amended claim 1 to make clear that the step of incubating occurs prior to staining.

Additionally, WO'655 teaches that "the first step in the cryopreservation method of the invention encompasses obtaining a previously selected sperm sample, as well as subjecting a source sample to any suitable selection method. Sperm from any species can be selected and frozen according to the method of the invention." *WO'655, page 5, lines 5-12.* WO'655 teaches that prior to staining "All samples were incubated at 5 °C for 24 or 48 hours post-collection." *WO '655, page 17, line 28.* It is well understood that the temperature above which sperm cell membrane lipids transition from a liquid phase to gel phase ("Tm") of claim 1 varies from species to species and that 5°C is well below the Tm of sperm cell membranes for most species. *See attached Exhibit C "Lipid Composition and Thermotropic Phase Behavior of Boar, Bull, Stallion, and Rooster Sperm Membranes." which evidences that for sperm cell membranes the Tm is above 5°C, page 255 at column 2, first paragraph; see also Publication at Paragraph [0003].* WO'655 does not provide any teaching that relates to the step of incubating semen at a temperature above which sperm cell membrane lipids transition from a liquid phase to gel phase. Applicant has amended claim 1 to make clear the temperatures at which sperm cells are incubated prior to staining. This amendment is supported by the specification at Paragraph [0038].

Additionally, the Examiner indicates that neither one of the WO'655 and the WO'906 teach staining for a period of 30 minutes. Applicant upon review of the WO '655 reference and the WO'906 reference, does not believe that there is any teaching in which the step of staining occurs for a duration of less than one hour. As such, Applicant has amended claim 1 to recite a period of time for staining of "about 25 minutes to about 50 minutes". This amendment is supported by the specification at Paragraph [0064] of the Publication.

Because neither of the WO'655 and the WO'906 teach the limitation of staining sperm cells for the period of time claimed, the Examiner cites Tardiff as teaching staining sperm cells with Hoechst 33342 for the claimed period of time. *Office Action at Page 5.*

However, with respect to the combination of WO'655 and Tardiff, in view of Applicant's above remarks all of the limitations of claim 1 are not still not taught as required

by *MPEP §2143 A (1)* because WO'655 does not teach "semen at a temperature above which sperm cell membrane lipids transition from a liquid phase to gel phase". Accordingly, Applicant respectfully requests that the Section 103 rejection based on the combination of WO'655 and Tardiff be withdrawn.

No Motivation to Combine References.

If the proposed modification or combination of the prior art would change the principal of operation of the prior art invention being modified, then the teaching of the references are not sufficient to render claims prima facie obvious" *MPEP Rule §2143.01 VI.*

With respect to the combination of WO'906 and Tardiff, there would be no motivation to combine the references. The WO'966 reference teaches that "the use of temperatures in the range of 30 °C to 39 °C in the presence of a QDVS [quantitative DNA vital binding stain] followed by ultraviolet laser based flow cytometry introduces a number of difficulties and disadvantages into the process which begins at semen collection and ends at fertilization which can reduce sperm viability and the efficiency (purity) of sorting sperm into GES [gender enriched semen]." *WO'906 at Page 2, lines 6-18.* Accordingly, WO'609 teaches "prolonged period of staining. . .at effective temperatures between about the thermotropic phase transition temperature T_m of the membranes of the sperm being sorted up to less than 30 °C . . .to reduce or eliminate the time required for higher temperature incubation with stain. The lower temperatures (compared to the prior art techniques) are also believed to provide advantageous effects on sperm orientation during sorting." *WO'609 at page 4, lines 28-33 and page 4, lines 1-5.*

By way of contrast, Tardiff teaches as to Experiment 2 (cited by the Examiner) and all other semen processing outside of Experiment 1 that frozen thawed semen are stained with Hoechst 33342 at 37 °C. With respect to Experiment 1 it appears that semen is mixed at 35 °C with TALP buffer (page 202, first column "Semen Extenders and Semen Processing") and then incubated for 30 minutes at 37 °C (page 202, second column, "Experiment 1").

Tardiff also states that "Although continuous exposure of fluorescently stained cells to UV illumination is often harmful. . .sperm are exposed very briefly. Furthermore, only a small subsample is used for analysis, and the sperm used for biological purposes are not exposed to the DNA stain." *Tardiff at page 205, second column.*

Modifying the WO'906 reference to include the semen handling and staining conditions taught by Tardiff would result in a substantially change to principle of operation of the WO'906 reference from that of maintaining sperm cells between the thermotropic transition temperature up to less than 30 °C and providing a staining period of not less about 1.5 hours (*WO'906, Table 2 1.5 hour incubation results in "close to separation)* to that of freezing sperm cells or establishing sperm cells at 35 °C and then staining for a period of 30 minutes at 37 °C (Tardiff Example 1 and Semen Processing).

Also, Tardiff does not stain sperm cells for biological purposes which is contrary to the teaching of WO'906 or WO'655 in which all sperm cells for biological purposes are necessarily stained.

Additionally, Tardiff stains sperm cells for the purpose of detecting sperm cells from non-sperm cell particles in media and not for the purpose of differentiating between X-chromosome bearing and Y-chromosome bearing sperm cells. A person of ordinary skill in the flow cytometry art would not find the Tardiff reference pertinent with respect to handling and preparing sperm cells for separation based upon the difference in the amount nuclear DNA.

Also, "If the proposed modification would render the prior art unsatisfactory for its intended purpose, then there is no motivation to make the proposed modification. *MPEP §2143.01 V.*

By introducing the semen handling and staining conditions taught by Tardiff into WO'906, would render the WO'906 reference unsatisfactory for its intended purpose of maintaining sperm cells in a narrow range of temperature throughout the semen handling and staining process.

Moreover, because Tardiff does not stain any sperm cells for biological purposes the combination would render both WO'906 and WO'655 unsatisfactory for their intended purpose of providing sex selected sperm cells for insemination/fertilization purposes.

Because the proposed modification would change the principal of operation or render the WO'906 and the WO'655 reference unsatisfactory for the intended purposes, a case of obvious cannot be established by the combination of WO'906 and Tardiff or the combination of WO'655 and Tardiff. Accordingly, Applicant respectfully requests reconsideration of claims 11, 4-12, 15, 16, 18, 21, 150, 151, 154, and 155 and withdrawal of the Section 103(a) rejection based on the combination of WO'906 and Tardiff and the combination of WO'655 and Tardiff.

Claimed Staining Time Is Not Mere Optimization. The Examiner indicates that Tardiff teaches that Hoechst 33342 is useful in flow cytometry methods. . .and that Johnson and Seidel teach staining of sperm cells with Hoechst 33342 for time periods of less than 1 hour in a separation method, thus. . .staining time and amount is mere optimization of results support the Examiner' position that staining time and amount is mere optimization. *Office Action at Page 11 and also at pages 6 and 10.*

Applicant respectfully disagrees, first the Seidel reference as above remarked should be removed as a reference because it was published after Applicant's priority date and cannot be utilized as a reference. Second, Johnson discloses a range which is not enabled as explained by the remarks which follow and therefore does not provide a teaching which can be used as evidence of a period of stain time. Third, Tardiff provides a general statement about the use of Hoechst 33342 stain as being useful in flow cytometry studies which in itself is not enabling with respect to the invention claimed nor discloses the general conditions of the claimed invention.

Typically a rejection due to optimization is made where the general conditions of a claim are disclosed in the prior art as a range in which result-effective variable is optimized.

MPEP 2144.05 II. In this case, the Examiner supports the rejection based on optimization with the Seidel reference which was published after the priority date of Applicant's application as above remarked, Johnson which is not enabled as below remarked, and general guidance from Tardiff which mischaracterizes its internal citations provided at page 205, column 1. In fact, the references cited by Tardiff of Evenson 1982; Graham 1990; Garner 1988; and Kramer 1993 do not even discuss any use of Hoechst 33342. *Copies of Evenson 1982; Garner 1988; and Kramer 1993 attached as Exhibits D, E, and F respectively.* The Morrell reference 1989 teaches staining with Hoechst 33342 overnight which is hours outside of the claimed range and at low temperatures outside of the claimed range before sorting. *Morrell, page 178, second column attached as Exhibit H.* The Morrell reference is further mischaracterized by Tardiff as not damaging to sperm cells. However, when the Morrel is read in its entirety it concludes "it is not possible to state categorically that sperm did not suffer from chromosomal damage in our system. . .It will be necessary to establish whether Hoeschst 33342 staining and/or flow cytometry does cause damage to sperm DNA before any widespread use is made of sperm treated in this manner." *Morrell, page 182, last paragraph attached as Exhibit H.*

All of which in combination do not disclose the general conditions of the claims in a manner which isolates "staining time" as being capable of mere optimization of a result effective variable. The Examiner's approach removes this limitation from other interrelated factors within the claimed invention which are effected by or effect stain time including sperm cell membrane temperature, staining temperature, the ability to determine the sex characteristic of a the sperm cells as a result of staining the sperm, the ability to separate the stained sperm cells based on occurrence of an X chromosome or a Y chromosome, and the biological purpose of the separated sperm cells.

For example, imposing the 30 minute stain time taught by Tardiff on the general conditions of the WO '906 reference yields an inoperative invention incapable of allowing sperm cells to separated by flow cytometry. Optimizing stain time within the WO '906 reference to maintain the ability to sort cells as claimed yields a stain time of not less than 1.5 hours based on the described general conditions. Imposing the stain temperature taught by

Tardiff of 37°C in the context of the WO'906 reference changes the basic premise of the WO'906 reference of prolonged staining at low temperature.

Accordingly, Applicant does not believe that the Examiner has established a case of optimization because the cited art for the various reasons explained does not provide the general conditions claimed in which the staining time can be optimized.

Supporting References Cited By Examiner. To cite a reference the reference must enable the claimed invention with sufficient clarity and detail to establish that the subject matter already existed in the prior art. *Elan Pharms. Inc. v. May Found. for Med. Education and Research, 304 F. 3d. 1221 (Fed. Cir. 2002).*

The claimed invention as amended limits staining to sperm cells which are incubated prior to staining at a temperature above which sperm cell membrane lipids transition from a liquid phase to gel phase at temperature between about 5°C and about 30 °C and staining the sperm cells with Hoechst 33342 stain at temperature in the range of about 30°C and about 35 °C for a period of time of about 25 minutes to about 50 minutes, determining the a sex charateristic of the sperm cells, and separating the sperm cells into X-chromosome and Y-chromosome bearing sperm cells.

The Johnson Reference. The Johnson reference is a review article which provides only general guidance that Hoechst 33342 can be used to stain sperm cells with the staining time from 0 minutes to 1 hour. *Johnson, page 100, first column.* There is no citation included in the reference to substantiate the range of 0 minutes to 1 hour. The guidance is so broad that the range taken as whole is inoperative. For example, it is clear that sperm cells cannot be stained at zero minutes and be separated as claimed (see WO'906 at Table 2, page 20 and Tardiff at page 202, Experiment 1) and at the other extreme staining for 1 hour has all the disadvantages which the claimed invention avoids by discovering a manner of staining sperm cells suitable for use with flow cytometry which takes much less time than 1 hour in

the precense of the other claimed limitations. Additionally, United States Patent No. 5,135,759 to Johnson ("Johnson '759") which as a US patent is presumed to be enabled for use of Hoechst 33342 stain in the context of flow cytometry with sperm cells states that "incubation for 1 hr at 35 °C was found to be acceptable but ranges of 30°C to 39°C would also be effective. Incubation time has to be adjusted according to temperature; that is 1.5 hrs for 30°C; 1 hr for 39°C." *Johnson, column 4, lines 37-43; See also, the WO'906 reference at page 2, lines 1-17.*

Johnson '759 Teaches Away. Johnson '759 also teaches away from the claimed invention and from Johnson and from Tardiff in that Johnson states that to stain sperm cells for separation by flow cytometry should be stained from "1 hr for 39°C".

Because the Johnson reference provides general guidance which is not enabling with respect to the claimed invention and there is a enabled reference by the same author/inventor which teaches away from or teaches contrary to the teaching relied upon by the Examineer, Applicant respectfully requests that the Johnson reference be withdrawn or given little weight.

Claims 5-12, 15, 16, 18, 21, 150, 154, and 155 are made ultimately dependent upon a base claim which Applicant believes is now in condition for allowance, accordingly each of claims 5-12, 15, 16, 18, 21, 150, 154, and 155 are also allowable. Any response is thereby made moot.

5. Request For Telephonic Interview. Applicant respectfully requests a telephonic interview with the Examiner to address any remaining concerns that the Examiner may have with respect to the Section 103 concerns.

CONCLUSION

Claims 2-4, 13-14, 17, 9-20, 22-149, and 152-153 have been without prejudice canceled. Claim 1 has been amended without the addition of any new matter. Applicant's amendment to claim 1 along with the above remarks overcomes the Section 103 concerns raised by the Examiner. Applicant believes that claim 1 as amended and all of the claims made ultimately made dependent on claim 1 are now in condition for allowance and Applicant respectfully requests allowance of same.

Dated this 22 day of March, 2010

Respectfully Submitted,

By: _____

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Articles:

☐ Seidel GE, Jr and DL Garner
Current status of sexing mammalian spermatozoa
Reproduction 2002 124: 733-743. [Abstract] [PDF]

☐ RA Fissore, M Kurokawa, J Knott, M Zhang, and J Smyth
Mechanisms underlying oocyte activation and postovulatory ageing
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Prolactin family miniarray: a tool for evaluating uteroplacental-trophoblast endocrine cell phenotypes
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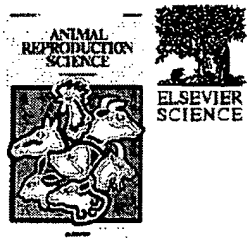

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CRYOBIOLOGY 29, 255–266 (1992)

Lipid Composition and Thermotropic Phase Behavior of Boar, Bull, Stallion, and Rooster Sperm Membranes

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Composition and thermotropic phase behavior of sperm membrane lipids from species ranging in sensitivity to cold shock were determined. Lipids from whole sperm and sperm plasma membrane were fractionated into neutral lipid, glycolipid, and phospholipid fractions. Compositional analyses were completed for free sterols, phospholipids and phospholipid-bound fatty acids. Phase transition temperatures were determined for phospholipid and glycolipid fractions using differential scanning calorimetry. Cholesterol was the major sterol in sperm lipids of all species. Cholesterol to phospholipid molar ratios were 0.26, 0.30, 0.36, and 0.45 for sperm plasma membrane of the boar, rooster, stallion, and bull, respectively. Choline and ethanolamine phosphoglycerides and sphingomyelin were the major phospholipid classes in sperm and their proportions differed across species. Phospholipid-bound fatty acyl compositions of choline and ethanolamine phosphoglycerides were characterized by a high proportion of docosapentanoyl and docosahexanoyl groups in mammalian sperm and shorter, more saturated groups in rooster sperm. Glycolipids represented less than 10% of total polar lipids for all species. Thin-layer chromatographic analysis indicated that the major glycolipid component of rooster sperm was different from that of mammalian sperm. Peak phase transition temperatures (T_m) for sperm membrane phospholipids were 24.0, 25.4, 20.7 and 24.5, for the boar, stallion, and rooster, respectively. Corresponding T_m's for glycolipids were 36.2, 42.8, and 33.4 with no exotherm for rooster sperm glycolipids. These results demonstrate a difference in both composition and thermotropic phase behavior of glycolipids between rooster and mammalian sperm which may be related to the greater tolerence of rooster sperm to rapid cooling.

The sensitivity of mammalian sperm to rapid cooling was recognized over 50 years ago by Milovanov (35) with cellular injury manifested in loss of selective permeability and integrity of the plasma membrane, outer acrosomal membrane, and mitochondria. These manifestations are accompanied by loss of motility, diminished metabolism, and release of intracellular and membrane constituents (see Watson and Morris (50) for review). Although the mechanism of cold shock injury has not been established, several lines of evidence suggest that damage during cooling may be related to thermotropic phase transitions in sperm membrane lipids.

Holt and North (20) observed aggregation of intramembranous particles in the plasma membrane overlying the postacrosomal region and tail of ram sperm slow-cooled to 5°C and attributed the aggregation to lateral phase separation of membrane phospholipids. Cold-induced clustering in the tail region was partially irreversible upon rewarming to 30°C. De Leeuw *et al.* (13) reported comparable results for bull and boar sperm cooled rapidly to 0°C, although particle redistribution was completely reversible when sperm were warmed to 38°C.

Brietbart and Rubenstein (3) observed a break in the Arrhenius plot of Ca^{++}-ATPase activity of ram sperm plasma membrane at 28°C, suggesting that a phase transition had occurred. A break in ATPase activity of ram sperm plasma membrane was also seen at 23°C by Holt and North (21).

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EXHIBIT C

Using fluorescence polarization, Holt and North (22) reported additional changes in molecular ordering at 17, 26, and 36°C in isolated ram sperm plasma membrane. Recently, Wolf *et al.* (53), using differential scanning calorimetry, detected major endothermic transitions in ram sperm plasma membrane and corresponding lipid extracts at 60 and 26°C. Canvin and Buhr (5) observed a thermotropic phase transition in boar sperm plasma membrane at 23°C. The occurrence of phase transitions have recently been reported in intact shrimp, human, goat, and boar sperm using Fourier transform infrared spectroscopy (9, 14) at temperatures corresponding to those at which cold shock injury would be expected.

Species differences in the susceptibility of sperm to cold shock are well established (39, 48, 50, 51), with rooster sperm being more resistant to damage during rapid cooling than mammalian sperm while boar sperm are more labile than sperm from other species (48, 49). These differences appear to be correlated with membrane lipid composition for mammalian species (49), as resistance to cold shock damage is greater for species with sperm membranes characterized by a high degree of saturation in the phospholipid-bound acyl moieties (40) and a high sterol to phospholipid ratio (11). The purpose of this study was to determine the relationship between sperm plasma membrane composition and phase behavior of membrane lipids in species with sperm ranging in cold shock sensitivity.

MATERIALS AND METHODS

Preparation of Sperm

Boar, bull, and stallion semen was obtained from males trained for collection with an artificial vagina. Rooster semen was obtained from males conditioned for collection by the massage technique (4). Boar and stallion semen was centrifuged at 500*g* for 15 min to pellet sperm and remove excess seminal plasma. Mammalian sperm samples were washed twice following a 6-fold dilution of semen (bull) or 10-fold dilution of sperm pellets (boar and stallion) with a modified Tyrode's solution (TS, containing NaCl, 100 m*M;* KCl, 3.1 m*M;* Na_2HPO_4, 0.3 m*M;* $NaHCO_3$, 24.9 m*M;* $MgCl_2$, 0.4 m*M;* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 10 m*M;* pH 7.4) followed by centrifugation at 500*g* for 10 min. Rooster sperm samples were washed by the same procedure following a 6-fold dilution with nonglycerolated Lake's medium (32).

Plasma Membrane Isolation

Plasma membrane was removed from sperm by nitrogen cavitation and isolated and purified by sucrose density gradient centrifugation as described previously (19, 37, 38). Washed sperm (2–5 × 10^9) were resuspended in 10 ml TS and equilibrated at 650 psi for 15 min on ice in a Kontes minibomb for nitrogen cavitation. Cavitated sperm were slowly extruded and centrifuged at 6000*g* for 10 min. Supernatants were recovered, the sperm pellet was resuspended in 10 ml TS, and the centrifugation was repeated. Supernatants were combined, applied to a discontinuous sucrose gradient (1.05, 1.14, and 1.19 g/ml), and centrifuged at 100,000*g* for 2.5 h in a Beckman SW28 rotor. Plasma membrane from all species was recovered at the 1.05/1.14 g/ml interface. These procedures provide preparations enriched in periacrosomal plasma membrane from boar and bull sperm (19, 38) and were used for preparation of plasma membrane fractions from other species without further characterization.

Lipid Extraction and Fractionation

Washed sperm and sperm membrane preparations were resuspended in Hepes-buffered saline (HBS; 0.15 *M* NaCl, 1.0 m*M* Hepes, pH 7.4) and aliquots were removed for protein determination (34) or

lipid extraction in 2:1 chloroform/methanol (18). Extracts were washed with theoretical upper phase, dried by rotary evaporation, and resuspended in 1% acetic acid in chloroform. Total lipids were fractionated by silicic acid chromatography (SepPak; Millipore, Milford, MA) according to Lynch and Steponkus (33).

Neutral lipids were eluted from SepPak cartridges with 12.0 ml of chloroform/acetic acid (100:1). Sterol composition of neutral lipid fractions was determined by gas–liquid chromatography (GLC) using an SP2250 column (Supelco, Bellefonte, PA) at 250°C. Peaks were identified based on retention times relative to purified standards and peak areas were integrated and quantified by computer using LabTech Chrom (IMI, State College, PA).

Glycolipids were eluted with 8.0 ml acetone followed by 8.0 ml acetone/acetic acid (100:1) and further fractionated by thin-layer chromatography (TLC) on silica gel H (0.25 mm, Analtech, Newark, DE) using a solvent system of chloroform/methanol/acetic acid/water (85:15:15:3).

Phospholipids were eluted with 10.0 ml chloroform/methanol/water (5:10:4). Water was removed from phospholipid fractions by adding 3.0 ml chloroform and 4.0 ml water to separate the eluent into an upper phase and a lower phase containing phospholipids. Phospholipids were further fractionated by TLC using a solvent system of chloroform/methanol/acetic acid/water (65:35:15:5) (47). Individual spots on TLC plates were identified by comparison with the R_f's of purified standards. Phospholipids were eluted from silica gel and water was removed from the eluent by phase partitioning as described above. Individual phospholipids were quantified by inorganic phosphate determination (1, 6).

Plasmalogen Separation and Analysis

Alkenyl-ether containing choline and ethanolamine phosphoglycerides, which are the major phosphoglycerides in sperm from several species, were separated from diacyl and alkylacyl species by two-dimensional TLC and acid treatment (23). Solvent was removed from TLC plates under a stream of nitrogen after TLC in the first dimension (47). Plates were then placed gel-side down 1.5–2.0 cm above a reservoir containing concentrated HCl for 10 min. Plates were purged with nitrogen until no HCl fumes were detected followed by development in the second dimension with chloroform/methanol/ammonia (100:50:12). Acid labile (plasmalogen) and acid stable spots were eluted and aliquot portions used for total phospholipid determination and fatty acid methyl ester analysis.

Preparation and Analysis of Phospholipid-Bound Fatty Acid Methyl Esters

Fatty acyl moieties of the major sperm phosphoglycerides were transesterified for GLC analysis. After removing solvent from samples under a stream of nitrogen, 1.0 ml methanolic base (Supelco) was added followed by incubation at 37°C for 30 min and extraction into diethyl ether. Each sample was supplemented with heptadecanoic acid methyl ester as a reference standard. Fatty acid methyl esters were analyzed by GLC using an SP2330 column (Supelco) at 175°C for 20 min increasing to 240°C at 6°C/min. Peaks were identified by retention times relative to authentic standards and verified by mass spectrometry. Peak areas were quantified by computer as described for sterols.

Differential Scanning Calorimetry of Sperm Membrane Lipids

Solvent was removed from 1–3 mg aliquots of sperm phospholipids or glycolipids under a stream of nitrogen and lipid samples were resuspended in a minimum volume of distilled, deionized water. Resuspended lipids were transferred to sample pans and analyzed by differential scanning calorimetry (Perkin-Elmer DSC-7). Peak

thermotropic phase transition temperatures (T_m's) were determined during scanning between 70 and −10°C at 10 or 20°C/min.

Statistical Analysis

Analyses of individual lipid fractions were completed on extracts of washed sperm and sperm membrane preparations from four separate pools of semen. Data were analyzed using the General Linear Models option of the Statistical Analysis System (45). Main effects in the model used were pooled ejaculates and species. Separate analyses were conducted for each lipid class. Percentage data were analyzed after arcsine transformation. Differences between means were tested by Duncan's multiple range test (15).

RESULTS

Component analyses of sperm plasma membrane fractions from different species are presented in Fig. 1. The average sperm membrane protein to phospholipid ratio was highest for boar sperm at 1.26 (w/w). Plasma membrane protein to phospholipid was lower for bull and stallion sperm (0.80 and 0.86, respectively) and very low for rooster sperm (0.46). Cholesterol was the major sterol present in sperm membranes from all species examined. The molar ratio of cholesterol to phospholipid was highest for bull sperm (0.45), lower for boar (0.26) and rooster (0.30), and intermediate for the stallion (0.36). Desmosterol was detected in mammalian but not rooster sperm lipids. When present, desmosterol was a minor sterol component, especially in bull sperm, and had little effect on the total sterol to phospholipid ratio.

Further analyses of sperm lipid fractions were completed on extractions of total sperm lipids. Phospholipid composition of sperm is presented in Fig. 2. Choline phosphoglycerides (CPs) were the predominant phospholipid fraction in sperm from all species. Bull and rooster sperm were characterized by a high ratio of CPs to ethanolamine phosphoglycerides (EPs) while boar and stallion sperm had a lower CP/EP ratio. Rooster sperm were also characterized by a higher percentage of phospholipid in the serine + inositol phosphoglyceride fraction than other species.

Plasmalogen content of CP and EP fractions from sperm of different species is presented in Fig. 3. Choline plasmalogen accounted for between 10 and 15% of total CP and EP in boar, stallion, and rooster sperm and over 55% in bull sperm. While total EP was lower than CP in all species, ethanol-

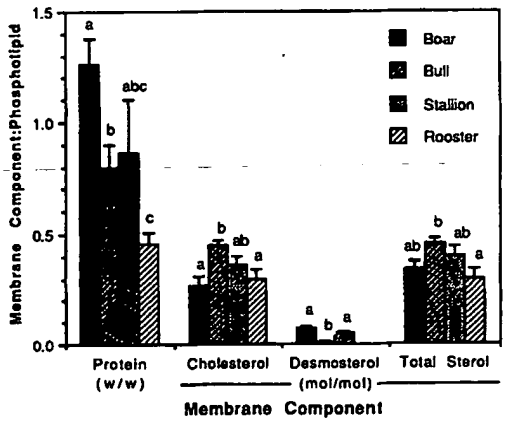

FIG. 1. Plasma membrane components of ejaculated boar, bull, stallion, and rooster sperm relative to membrane phospholipid ($\bar{X}$ ± SEM, n = 4). [a,b,c]Means with different superscripts are different ($P < 0.05$).

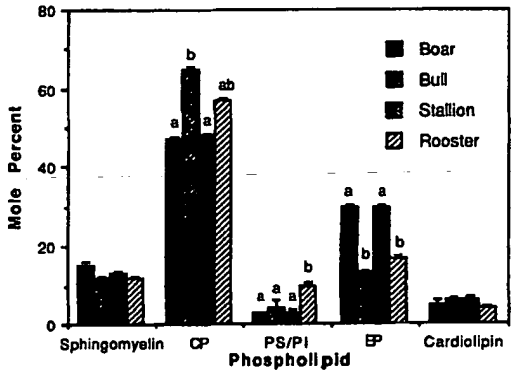

FIG. 2. Major phospholipids of boar, bull, stallion, and rooster sperm. CP, choline phosphoglycerides; PS + PI, serine and inositol phosphoglycerides; EP, ethanolamine phosphoglycerides ($\bar{X}$ ± SEM, n = 4). [a,b]Means with different superscripts are different ($P < 0.05$).

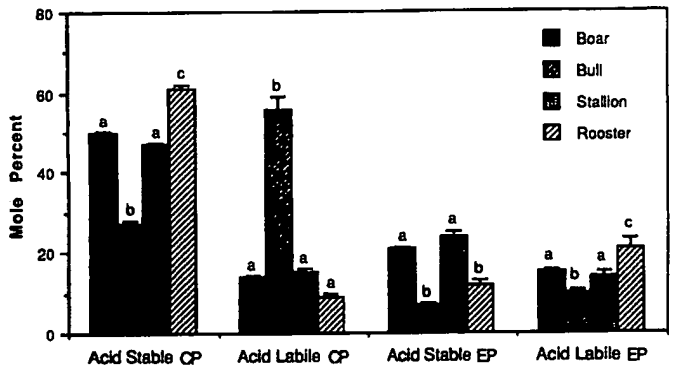

FIG. 3. Acid stable (diacyl and alkylacyl) and acid labile (alkenylacyl or plasmalogen) content of sperm CP and EP fractions. Values are expressed as the mole percentage of total CP and EP ($\bar{X}$ ± SEM, n = 4). [a,b]Means with different superscripts are different ($P < 0.05$).

amine plasmalogens made up 35% or more of the EP fractions in all species examined.

Fatty acyl moieties of total sperm CPs are presented in Fig. 4. CPs from mammalian sperm were characterized by a very high proportion of docosapentanoyl and docosahexanoyl chains, with 22:5 predominant in boar and stallion sperm and 22:6 highest in bull sperm. Fatty acyl groups of rooster sperm CPs meanwhile, were more broadly distributed in chain length and degree of unsaturation, with a higher proportion of shorter, more saturated species (16:0, 18:0, 18:1). Acid stable CPs, which include diacyl and alkylacyl species (Fig. 5), had fatty acyl profiles similar to total CPs for each species, with a slight decrease in polyunsaturated chains and corresponding increase in more saturated fatty acyl groups in the boar, stallion, and rooster. Acyl moieties of choline plasmalogens (Fig. 6) from mammalian sperm were predominantly 16:0, 18:0, 22:5, and 22:6 with little or no intermediate chain lengths. Acyl groups for bull and stallion choline plasmalogens were almost entirely 22:5 and 22:6. The fatty acyl profile for rooster sperm choline plasmalogen was similar to that for total and acid stable CPs.

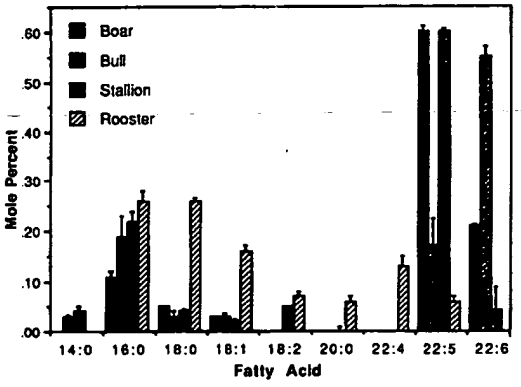

FIG. 4. Fatty acyl moieties of total choline phosphoglycerides from sperm of different species. Values are presented as a mole percentage of total fatty acid methyl ester quantified ($\bar{X}$ ± SEM, n = 4).

Total sperm EPs also contained a high proportion of long chain, polyunsaturated fatty acyl groups (Fig. 7), especially 22:5 and 22:6 in mammalian sperm and 22:4 in rooster sperm. A lower proportion of polyunsaturated chains was measured in acid stable EPs of all species except the stallion (Fig. 8). Acid stable EP from bull sperm also contained a very high level of arachidonic acid. Acyl chain distribution for ethanolamine plasmalogens (Fig. 9) was similar to that for total EPs with fewer intermediate-length chains in mammalian sperm.

Based on limited observations, the glycolipid content of sperm was estimated to be 5–8% of total polar lipid for mammalian species (bull and boar) and 4–6% for the rooster. Separation of glycolipids by TLC demonstrated that sperm from all species contained a single, dominant glycolipid component (Fig. 10). For mammalian sperm, this component migrated with an R_f

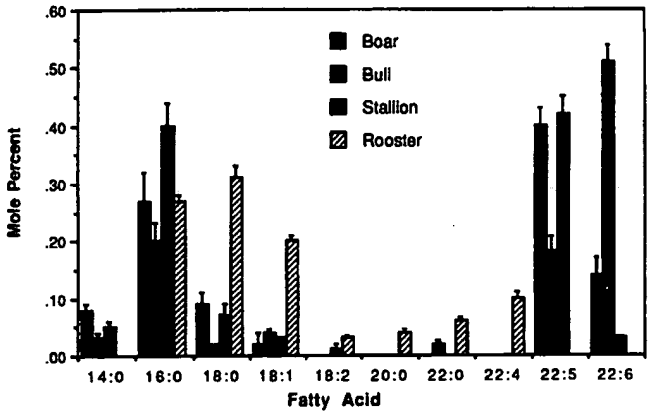

FIG. 5. Fatty acyl moieties of acid stable choline phosphoglycerides from sperm of different species. Values are presented as a mole percentage of total fatty acid methyl ester quantified ($\bar{X} \pm$ SEM, $n = 4$).

of 0.16. The major glycolipid present in rooster sperm migrated with an R_f of 0.06.

Representative DSC thermograms for total boar and rooster sperm phospholipids are presented in Fig. 11. Exotherms corresponding to thermotropic phase transitions were recorded during cooling from 70 to −10°C. The average T_m's for sperm phospholipids were similar for all species (Table 1).

Thermograms for glycolipids from boar and rooster sperm (Fig. 12) demonstrate a marked difference in phase behavior between the glycolipid fractions from these species. A broad exotherm occurred between 40 and 45°C for boar sperm glycolipids but no exotherm was observed during scanning of glycolipids from rooster sperm. The T_m for boar sperm glycolipid was intermediate to those for bull and stallion sperm (Table 1).

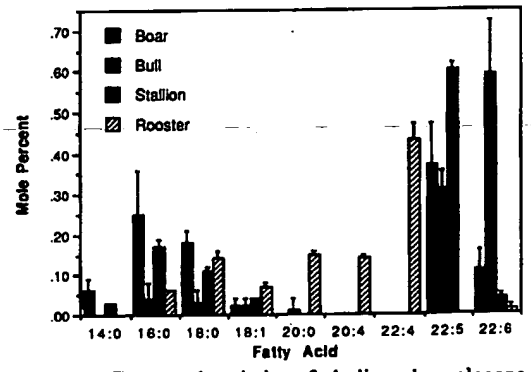

FIG. 6. Fatty and moieties of choline plasmalogens from sperm of different species. Values are presented as a mole percentage of total fatty acid methyl ester quantified ($\bar{X} \pm$ SEM, $n = 4$).

DISCUSSION

The lipid composition of total sperm and sperm plasma membrane determined in this study are in good general agreement with other published reports for the species examined (see Table 2), confirming major species differences in the molecular features of sperm membrane lipids. However, the molecular configuration which confers cold stability is not readily apparent. Even though large species differences in phospholipid and phospholipid-bound acyl groups of sperm were measured, relatively little species variation was observed in peak thermotropic phase transition temperatures. Peak transitions occurred between 20 and 25°C with no obvious relationship to cold shock sensitivity. These transition temperatures correspond closely to those for sperm of related species, including 17–28°C for ram sperm plasma membrane (3, 21, 22, 53), 18–24°C for boar sperm (5, 9, 14), and 21°C for goat sperm (9, 14).

Absence of a clear correlation between

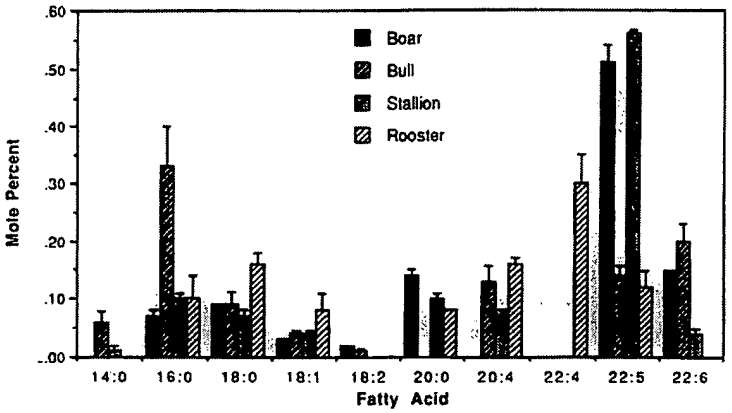

FIG. 7. Fatty acyl moieties of total ethanolamine phosphoglycerides from sperm of different species. Values are presented as a mole percentage of total fatty acid methyl ester quantified ($\bar{X} \pm$ SEM, $n = 4$).

T_m's of sperm phospholipids and cold shock sensitivity for different species suggests that differences in other components in the native membrane (sterols, proteins and glycolipids) must modulate the occurrence or consequences of phase transitions in ways which alter membrane stability and function during cooling. For example, the phase broadening effect of cholesterol on membrane phospholipids during cooling and consequently on membrane permeability characteristics and enzyme activity is well established (2, 54) and a positive correlation between sperm cholesterol to phospholipid molar ratios and cold shock resistance has been suggested for mammalian sperm (11). This relationship was not observed for the more resistant rooster sperm, however.

Also, a relationship between cold shock sensitivity and protein content of the plasma membrane was apparent in this study, with the more sensitive sperm of the boar having a higher protein content than other mammalian species and rooster sperm plasma membrane having a low protein content. The effects of phase separation on membrane protein distribution dur-

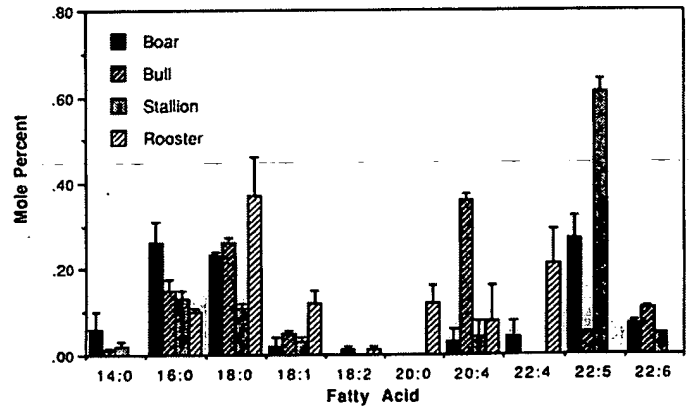

FIG. 8. Fatty acyl moieties of acid stable ethanolamine phosphoglycerides from sperm of different species. Values are presented as a mole percentage of total fatty acid methyl ester quantified ($\bar{X} \pm$ SEM, $n = 4$).

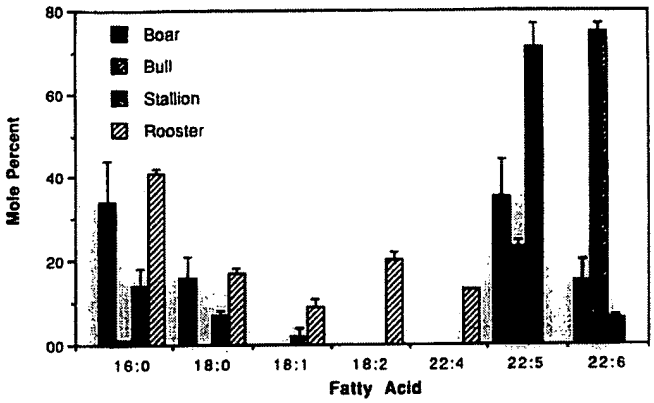

FIG. 9. Fatty acyl moieties of ethanolamine plasmalogens from sperm of different species. Values are presented as a mole percentage of total fatty acid methyl ester quantified ($\bar{X} \pm$ SEM, $n = 4$).

ing slow cooling are generally considered to be reversible. However, during rapid cooling, packing faults and other perturbations created by the formation of multiple gel phase domains may be exacerbated by proteins (43). Thus, structural defects may increase with increasing protein, leading to altered permeability and potentially damaging effects to the cell (51).

Furthermore, Quinn (44) has suggested that nonbilayer preferring phospholipids (as ethanolamine phosphoglycerides) interact preferentially with integral membrane proteins or are constrained to a bilayer configuration by proteins and other membrane components at physiological temperatures. Because of their generally higher liquid crystalline to gel transition temperatures, nonbilayer preferring lipids may be converted into a bilayer configuration during cooling, thereby altering protein functions dependent upon interactions with nonbilayer lipids (44). Nonbilayer preferring phospholipids might also become sequestered during cooling below their liquid crystalline to gel phase transition temperature as a consequence of lateral phase separa-

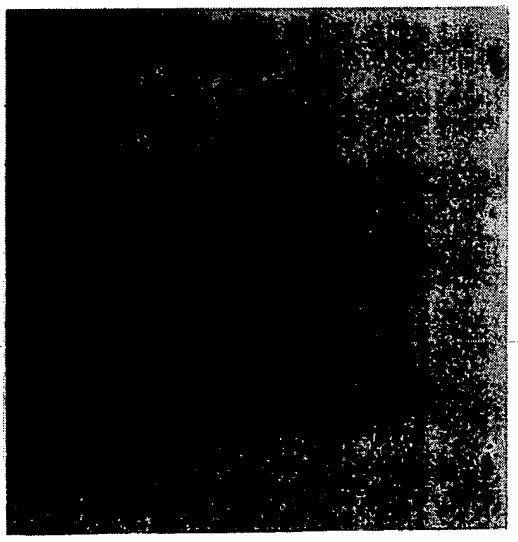

FIG. 10. Thin layer chromatogram of sperm glycolipids from boar (Bo), rabbit (Ra), bull (Bu), stallion (St), and rooster (Ro) sperm. Each lane represents the glycolipid fraction from a separate sperm preparation.

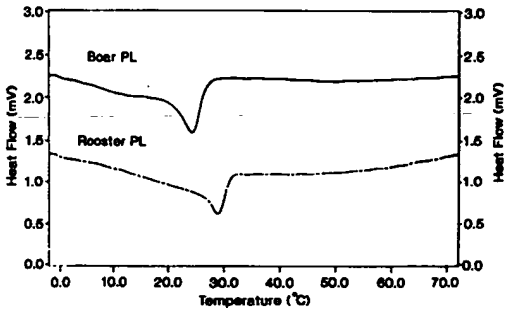

FIG. 11. Representative DSC thermograms of total sperm phospholipids from boar and rooster sperm scanned between 70 and −10°C. Exotherms correspond to thermotropic phase transitions of the dispersed lipid.

TABLE 1
Peak Thermotropic Phase Transition Temperatures (T_m) for Phospholipids and Glycolipids of Sperm from Different Species

Species	T_m (°C)	
	Phospholipids	Glycolipids
Boar	24.0 ± 0.5	36.2 ± 2.8
Bull	25.4 ± 1.1	42.8 ± 1.2
Stallion	20.7 ± 0.8	33.4 ± 1.4
Rooster	24.5 ± 3.9	Transition not detected

Note. $\bar{X}$ ± SEM for lipid fractions from two to three separate sperm samples.

tion. Upon rewarming, there would no longer be constraints on the phase-separated lipids to prevent formation of nonbilayer, membrane-perturbing structures (43, 44) which could lead to membrane disruption. Based on this theory, the relatively high ethanolamine phosphoglyceride content of boar sperm plasma membrane may be required to accomodate the higher protein content of the plasma membrane, thus increasing the probability of altered protein function and bilayer perturbations during cooling and rewarming.

Striking species differences were also observed for composition and phase behavior of sperm glycolipids. Some species variation in the T_m's of glycolipids from mammalian sperm was measured, but no direct correlation to cold shock sensitivity was apparent. Rooster sperm glycolipid, however, exhibited no phase transition in the temperature range examined. The relatively high transition temperatures for mammalian sperm glycolipids are consistent with molecular species having saturated hydrocarbon chains. Glycolipid composition was not determined in this study, but others have determined that the principal glycolipid in boar and bull sperm is 1-0-alkyl-2-0-acyl-3-sulfogalactosyl glycerol (25, 36, 46) containing almost exclusively 16:0 alkyl and acyl moieties (36, 46). Rooster sperm glycolipids, to our knowledge, have not been characterized previously. Chemical and physical characteristics measured in this study suggest that the hydrocarbon moieties may be more unsaturated than in mammalian sperm, although compositional analyses have not been completed.

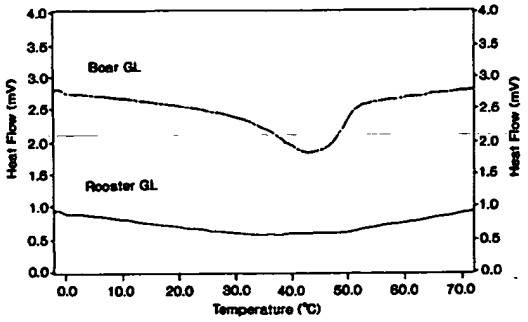

FIG. 12. Representative DSC thermograms of total sperm glycolipids from boar and rooster sperm scanned between 70 and −10°C. The exotherm in the thermogram for boar sperm glycolipids corresponds to the phase transition of the dispersed lipid. No transition was observed for rooster sperm glycolipid.

Because of their high liquid crystalline to gel transition temperatures, mammalian sperm glycolipids may contribute to the lateral phase separation phenomena reported for the bull, ram, and boar (13, 20). Recently, Wolf *et al.* (53) reported that ram sperm plasma membrane vesicles and corresponding lipid extracts exhibited two major endothermic transitions during DSC, suggesting the coexistence of liquid crystalline and gel phase lipids at physiological temperatures. The transition centered around 26°C corresponds to transition temperatures for sperm phospholipids in the present study. A second transition was observed near 60°C and was tentatively attributed to the presence of disaturated phospholipids or glycolipids. In addition to phase transitions in ram sperm plasma membrane around 17 and 26°C, Holt and North (22) reported a third transition between 35–38°C which corresponds closely to phase transition temperatures for mammalian sperm glycolipids reported here. Segregation of glycolipids as a result of

TABLE 2
References for Published Reports Describing Lipid Composition of Boar, Bull, Stallion, and Rooster Spermatozoa

Lipid fraction	Species			
	Boar	Bull	Stallion	Rooster
Phospholipid	8,10,17,26,28,36[a],52	7,10,38[a],42,46,52	29	12,24
Phospholipid-bound hydrocarbons	16,27,28,36[a],41	40,41,42,46		12
Sterols	16,30,36[a]	11,31,38[a],42	29	
Glycolipids	25,36[a]	46		

[a] Composition for plasma membrane.

phase separation during cooling could be potentially damaging if constraints on the formation of nonbilayer configurations are removed during rewarming, as with the monogalactosyldiglycerides of plant chloroplasts (44).

The difference in phase behavior between mammalian and rooster sperm glycolipids suggests greater overall fluidity of rooster sperm membranes at physiological temperatures. Absence of the high melting point glycolipid component in rooster sperm may limit chilling-induced phase separation effects and confer greater cold stability. The apparent relationship between sperm glycolipid composition and cold shock has not been recognized previously, to our knowledge. Glycolipids are localized in the plasma membrane, which is the primary site of cold shock injury. Thus additional characterization of glycolipid content and composition of sperm plasma membrane from species differing in cold shock sensitivity and determining the influence of glycolipids on the phase behavior of other membrane components may provide additional insight into the molecular mechanism of cold shock damage.

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Letters to the Editor

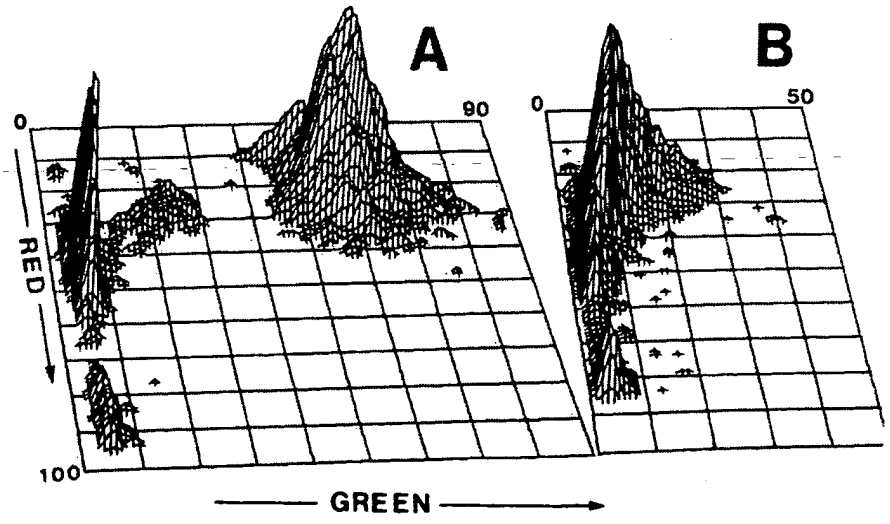
Simultaneous Measurement by Flow Cytometry of Sperm Cell Viability and Mitochondrial Membrane Potential Related to Cell Motility[1]

Flow cytometry of sperm cells stained green with rhodamine 123 (R123) and red with ethidium bromide (EB) provides a rapid, quantitative analysis of mitochondrial membrane potential (correlated here with cell motility) and cell viability. The staining specificity of R123 for mitochondria was described recently by Johnson et al. (5) and quantitated via flow cytometry by Darzynkiewicz et al. (2,3). After staining with R123, differences in fluorescence intensity between cells are believed due to a variable number of mitochondria per cell (2) or a difference in mitochondrial membrane potential (1,6), both of which are related to the metabolic processes of the cell. Ethidium bromide is used as a counterstain to determine cell viability, indicated by exclusion of the dye by living cells.

The sperm in suspension, stained with R123 and EB, were measured individually in a flow cytometer at rates up to one thousand per second. Thus statistically significant populations were easily assayed, offering an advantage over the slower, semiquantitative methods used currently for analyses of sperm motility and viability. While the EB dye exclusion technique for assessing cell viability is well-established, we believe this is the first report of a sperm "motility" assay based on measurements of mitochondrial membrane potential.

Sperm in suspension, stained with R123/EB as described in the caption to Figure 1, were observed by ultraviolet and light microscopy using a Leitz Orthoplan microscope fitted with epifluorescent illumination (485 nm excitation and 530 nm emission filters). The green fluorescence of R123 was restricted to the midpiece containing the mitochondria, and the red fluorescence of EB staining putative dying

Figure 1. Two parameter frequency histograms demonstrating stainability of human sperm cells with rhodamine 123 and ethidium bromide. A single semen sample obtained from a normal individual of proven fertility was stained and measured at (A) 4 hr after collection, and (B) 30 hr after collection. The sample was kept at room temperature in a flat-bottomed plastic jar. Prior to measurement, the semen was diluted to approximately $1–2 \times 10^6$ cells per ml in RPMI 1640 media (GIBCO, Grand Island, NY) containing 10% fetal calf serum (GIBCO). Alternatively, samples have been suspended and stained in phosphate buffered salt solution (PBS) containing 1% (w/v) sucrose with similar results. The samples were stained with R123 (Eastman Organic Chemicals, Rochester, NY, laser dye purity) at 10 μg/ml final concentration for 10 min; then the sperm were pelleted, resuspended in the same medium, and counterstained with EB (10 μg/ml, Polysciences, Warrington, PA). A stock solution of R123 was made in distilled water (1 mg/ml) and diluted into the sample solution. All procedures were done at room temperature. After 30–60 min the samples were analyzed in a FC 200 Cytofluorograf equipped with a 488 nm argon-ion laser (Ortho Diagnostic Instruments, Westwood, MA) interfaced to a Data General minicomputer. The green (F_{530} at 515–575 nm) and red ($F_{>600}$ measured in a band of 600–650 nm) fluorescence emissions are separated optically and measured by different photomultipliers; the integrated values of the respective pulses of each cell are recorded by the computer. The data are based on the measurement of 5,000 cells.



[1]Supported by National Institutes of Health and National Cancer Institute Grant HD12196 NCI Grant CA28704 and CA08748.

EXHIBIT D

or dead cells was located in the sperm head. Bright green fluorescence was correlated with vigorous sperm motility, while dull green fluorescence correlated with slow motility. Bright red and green fluorescence were mutually exclusive. However, some cells did exhibit both pale red and pale green fluorescence localized in the head and midpiece, respectively. These and other data indicated that impaired or dying cells stained with R123 under nonequilibrium conditions lose green fluorescence before, or as, they begin to show red fluorescence. Somatic cells undergo a transient increase in uptake of R123 prior to cell death and lysis (3), not seen in dying sperm cells.

Figure 1 shows data on a single ejaculate of sperm cells obtained from a fertile male; sample A was measured 4 hr after collection and sample B at 30 hr. At 4 hr, 70% of the sperm had a high level of R123 staining and 26% had a reduced level; 4% had a high level of EB staining, indicating dead cells. Thirty hours after collection, all cells had a reduced though variable level of R123 staining relative to sample A; 13% had a high level of EB staining. Light microscope observations showed a high level of motility in the 4 hr sample and a low level of motility in the 30 hr sample. This experiment was repeated with three different samples with similar results. Samples obtained from clinical patients, which showed reduced sperm motility under the light microscope when compared to control specimens from fertile subjects, had a lower level of R123 staining and a higher level of EB staining (Evenson et al., manuscript in preparation). These observations suggest that the changes in R123 fluorescence intensity in ejaculated sperm are primarily due to changes in mitochondrial membrane potential rather than mitochondrial number.

The staining intensity of R123 is concentration dependent under equilibrium conditions over a range of 0.2–150 μg/ml; staining at 10 μg/ml as used here provides for a strong fluorescent signal. The uptake of the dye is rapid, reaching a plateau by 10 min; after transfer of the cells to dye-free media a new equilibrium is reached by 30 min and remains stable for at least 1 hr.

These data and studies of other patients with known fertility problems show good correlation between motility of sperm by light microscopy and intensity of R123 staining (manuscript in preparation). This new technique for simultaneously quantitating sperm "motility" and viability by flow cytometry offers objective measurements based on large numbers of cells per sample. These measurements alone or in combination with the methods recently described by us (4) correlating fertility with nuclear chromatin resistance to denaturation in situ, demonstrate the usefulness of flow cytometry in the infertility clinic.

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FLUOROMETRIC EVALUATION OF CRYOPRESERVED BOVINE SPERMATOZOA EXTENDED IN EGG YOLK AND MILK

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ABSTRACT

A comparison of fluorogenically quantifiable parameters of cryopreserved, bovine spermatozoa that had been processed in homogenized milk and egg yolk citrate-based extenders was made using flow cytometry. Semen from four bulls was processed in egg yolk-citrate or homogenized milk extenders, packaged in straws and frozen at -196°C. Samples were thawed at 37°C, subdivided into three portions and stained after 0, 1.5 and 3 h of incubation at 37°C. Spermatozoa were stained using a combination of carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) and analyzed by dual parameter flow cytometry. The sperm cells were quantified fluorometrically at each time interval for both green and red fluorescence. The proportion of spermatozoa retaining the fluorescent CFDA derivative was larger at each time interval for samples in egg yolk citrate than those in milk. Differences in the retention of spermatozoal viability were detected between identical samples of bovine spermatozoa extended in milk or egg yolk based media.

Key words: bull, semen, fluorescence, milk extender

INTRODUCTION

Supravital stains or "live-dead stains" are not effective in assessing viability of cryopreserved spermatozoa because glycerol, a necessary ingredient in most cryopreservation media, interferes with staining (1). This problem, along with those presented by opaque media, can be overcome by using fluorescent stains (2). Combinations of fluorogenic stains such as carboxyfluorescein diacetate

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EXHIBIT E

(CFDA) and propidium iodide (PI) are powerful tools in assessing the functioning of spermatozoa (4-7). The CFDA, a membrane-permeant, colorless enzyme substrate, is rapidly converted by intracellular esterases into a green fluorescent derivative, which is retained within the membrane of functional cells because it is relatively membrane impermeant (6,8). The PI stains the nuclei of degenerative spermatozoa red but viable cells remain stain free (6). Although these fluorophores can be used effectively to microscopically assess viability of spermatozoa stored in opaque media, they are very labor intensive. Rapid assessment of spermatozoan viability is possible using fluorescent probes and an automated analytical system such as flow cytometry (3). Flow cytometry has the capability of not only rapidly quantifying specific characteristics of single sperm cells, but it also can compile information from a much larger number of individual spermatozoa. Although the utility of flow cytometric analyses for assessing the functional aspects of bovine and boar spermatozoa stored in egg yolk-based media has been demonstrated (4-7), similar analyses of spermatozoa stored in homogenized milk-based extenders are yet to be established.

The objectives of this study were 1) to determine if functional parameters of cryopreserved bovine spermatozoa processed and stored in milk-based extenders could be evaluated using fluorogenic staining and flow cytometry and 2) to quantify and compare functional parameters of duplicate samples of cryopreserved bovine spermatozoa that had been processed and stored in milk- and egg yolk-based extenders using fluorogenic staining and flow cytometry.

MATERIALS AND METHODS

Semen Samples

Ejaculates from four dairy bulls in routine semen production were split sampled and processed in milk-based (9) extender and in standard 20% egg-yolk-citrate medium (10). The diluted ejaculates were examined for spermatozoal concentration, total spermatozoal numbers and percentage of progressively motile spermatozoa before packaging in 0.5-ml polyvinylchloride straws. Groups of eight straws from each of the paired samples were frozen in liquid nitrogen vapor using standard cryopreservation procedures (11) and stored at -196°C. Postthaw examinations were done immediately in a composite sample from three straws thawed at 37°C for 60 sec.

Dual Fluorogenic Staining

Each straw, whether containing spermatozoa cryopreserved in milk- or egg yolk-based media, was thawed and diluted with two volumes of Beltsville thawing solution (BTS; 11). These samples were then subdivided into three portions, one stained immediately, another after 1.5 h of incubation and the remainder after 3 h of incubation at 37°C. Each aliquot (0.5 ml) was differentially stained with a combination of CFDA and PI (Source: Calbiochem, La Jolla, CA). The fluorogenic substrate, CFDA (2 ul, 4mg/ml DMSO), was added to each semen aliquot and mixed by careful repetitive pipetting and inverting the sample tube three times. Each aliquot was incubated at 37.5°C for 15 min before the nucleic acid-specific stain, PI (25 ul, 1.0 mg/ml in 0.1 M phosphate buffer, pH 7), was added. Each aliquot was carefully mixed and incubated at 37.5°C for another 15 min to insure uniform staining before microscopic and flow cytometric analyses. Cytometric analyses were replicated four times.

Microscopic Examination

Fluorescent staining of the spermatozoa was evaluated with an epi-fluorescence Zeiss Universal microscope equipped with a fluorescein isothiocynate (FITC) filter set (BP 485/20 excitation filter, FT 510 dichromatic beam splitter and a LP 520 barrier filter). A 4-ul aliquot of each sample was pipetted onto a clean slide and covered with a 24 x 50-mm coverglass to form a very thin specimen (6).

Flow Cytometry

Spermatozoa that were differentially stained with the combined CFDA and PI fluorogenic stains were quantified with an EPICS V Flow Cytometer (Coulter Electronics, Inc., Hialeah, FL). The stained cells were excited at 514 nm using a 5-watt, argon-ion laser (Coherent, Inc., Palo Alto, CA). Each analysis consisted of a minimum of 100,000 stained spermatozoa, which were each quantified simultaneously for green and red fluorescence at 200 to 300 cells/sec. Filter arrangements were 540 nm long pass interference and 530 nm long pass absorbance blocking filters; a 590 nm long pass absorbance for red fluorescence; and a 560 nm short pass for green fluorescence. All spermatozoa were measured simultaneously using this arrangement. This dual parameter system identifies three major spermatozoal populations designated A, B and C (Figure 1). These three populations of stained spermatozoa were quantified using the QUADSTAT Analysis program (Coulter Electronics, Inc., Hialeah, FL). Two separate quadrant analyses were utilized for each sample to quantitate spermatozoal populations A, B and C (Figure 2).

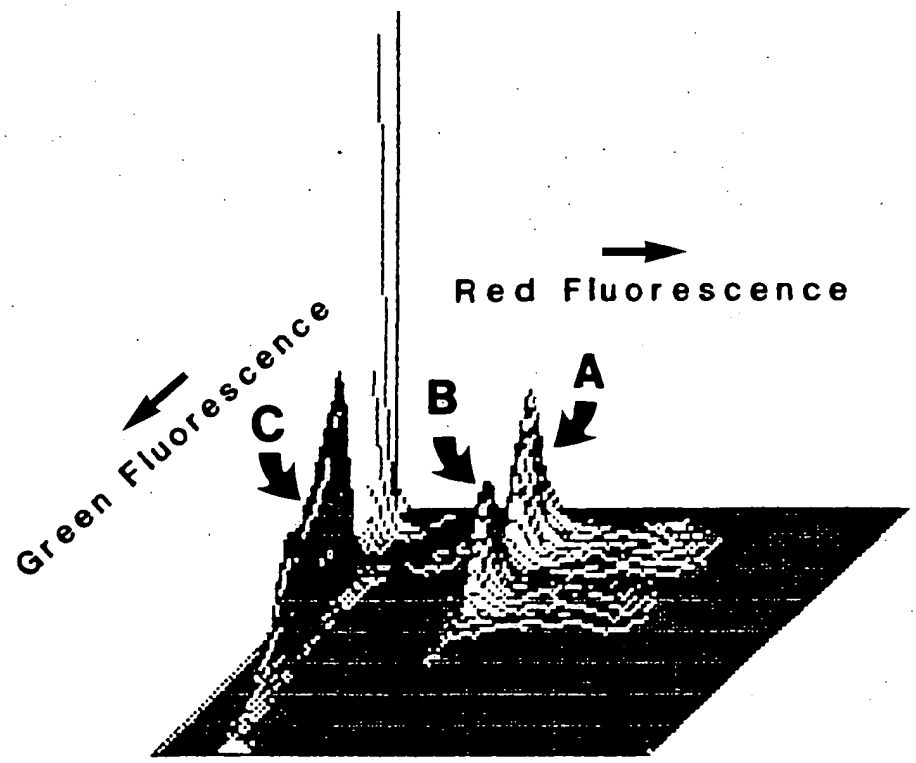

Figure 1. A three-dimensional histogram display showing the three spermatozoal populations A, B and C as identified by fluorogenic staining with carboxyfluorescein diacetate and propidium iodide and quantified by flow cytometry.

Data analyses

The populations of spermatozoa, which were identified and quantified by the Quadrant Statistic Analysis system, were analyzed using the Statistical Analysis System (SAS; 12) for ANOVA and correlation coefficients. Duncan's New Multiple Range Test was used to determine differences between extenders and time intervals.

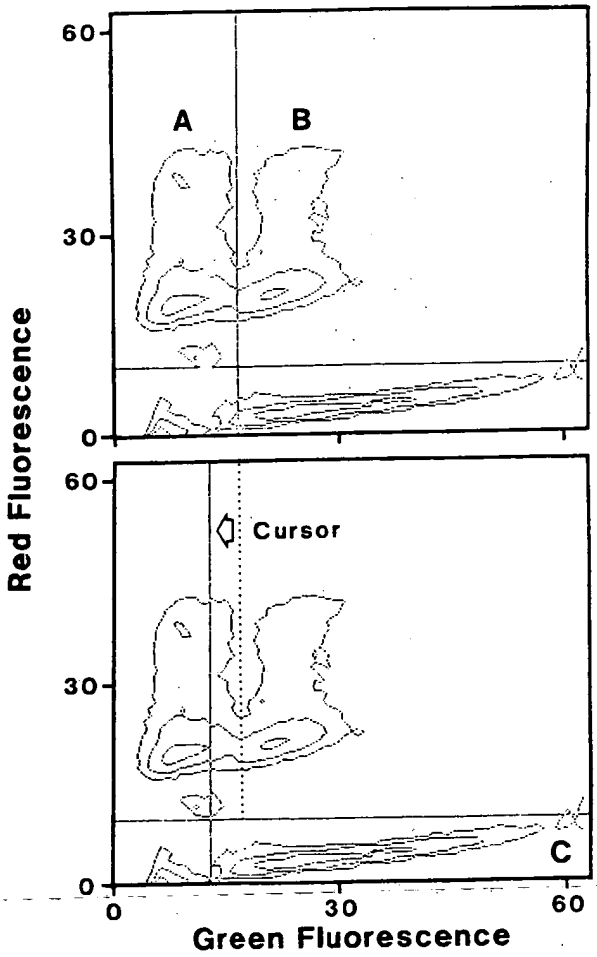

Figure 2. An example of quadrant analysis of spermatozoal populations A, B and C identified by fluorogenic staining with carboxyfluorescein diacetate and propidium iodide and measured by flow cytometry. The three populations of stained spermatozoa were quantified using the QUADSTAT Analysis program. Two separate quadrant analyses were utilized for each sample (note cursor line movement shown by arrow). This sample was composed of 91,528 stained spermatozoa. The number and percentage of spermatozoa in populations A, B and C were 34,900, 38%; 26,427, 29%; and 30,201, 33%, respectively.

RESULTS

Cryopreserved bovine spermatozoa in homogenized milk extender were readily examined for functional parameters using dual fluorogenic staining. Three distinct populations of spermatozoa in samples of both milk- and egg yolk based extenders were identified using microscopic examinations and flow cytometric analyses. An example of the quadrant analysis used to determine the number of spermatozoa in each of the three populations is shown in Figure 2. Population A was considered to be dead cells because PI readily stained the nuclei of these cells red. Population B cells were also stained by PI, but they retained some of the CFDA derivative in their mitochondria and/or acrosome. The quadrant lines were adjusted to determine the number of spermatozoa retaining the CFDA derivative throughout the cytoplasm and organelles of the cell. This population, identified as C (Figure 2), was considered to have functional membranes because of the retained fluorophore. These green-stained cells were considered to be viable as previously concluded from data derived from digitonin treatment and swim-up samples (6).

Dual flow cytometric analysis of the duplicate samples stored in either milk- and egg yolk-based extenders demonstrated that the proportion of spermatozoa retaining CFDA (Population C) was greater in egg yolk than in milk-based extenders (Table 1, $P<0.0001$). Incubation resulted in a decrease in population C (Table 1, $P<0.0001$), and significant differences ($P<0.0001$) among bulls in the proportion of spermatozoa retaining the CFDA were found. All three spermatozoal populations were highly correlated with the type of media used (Table 2, $P<0.01$). The increases that occurred in population A during incubation are reflected by the positive correlation with time intervals ($r = 0.41$, $P<0.01$). Likewise, the observed decreases in populations B and C are reflected by negative correlations ($r = -0.36$ and -0.35, $P<0.01$). The average for the four bulls in the proportion of spermatozoa retaining the CFDA derivative was larger ($P<0.05$) at all three time intervals in egg yolk-based samples than in milk (Figure 3). The rate of decrease in CFDA retaining spermatozoa (Population C) was similar over the 3-h incubation period for samples, whether processed in homogenized milk- or in egg yolk-citrate extender (Figure 3).

Table 1. Percentage of spermatozoa retaining green fluorescence (Population C) as determined by dual parameter flow cytometric analysis of bovine semen of four bulls that had been extended and cryopreserved in egg yolk-citrate and homogenized milk media[a]

Incubation Time (hour)	Bull A		Bull B		Bull C		Bull D	
	Egg	Milk	Egg	Milk	Egg	Milk	Egg	Milk
0	64	41	60	44	53	33	68	44
1.5	59	38	58	39	52	31	68	46
3.0	48	25	49	34	39	26	56	36

[a]Means of four replications of flow cytometric analyses of the fluorogenically stained spermatozoa using 10,000 cells per analysis. Replications were not different ($P>0.05$). Differences in bulls, media and incubation time were found ($P<0.0001$).

Table 2. Correlation coefficients (r values) among bulls, incubation times and the fluorogenically identified populations of bovine spermatozoa cryopreserved in egg yolk-citrate and homogenized milk media[a]

Sperm Population[a]	Bull	Extender	Time	Replication	Population[a] C	Population[a] B
A	-0.07	0.83**	0.41**	0.02	-0.95**	-0.69**
B	0.14	0.62**	-0.36**	0.01	0.43**	
C	0.03	0.77**	-0.35**	-0.03		

[a]The flow cytometric analyses of the fluorogenically stained spermatozoal populations A, B and C were replicated four times.
**$P<0.01$.

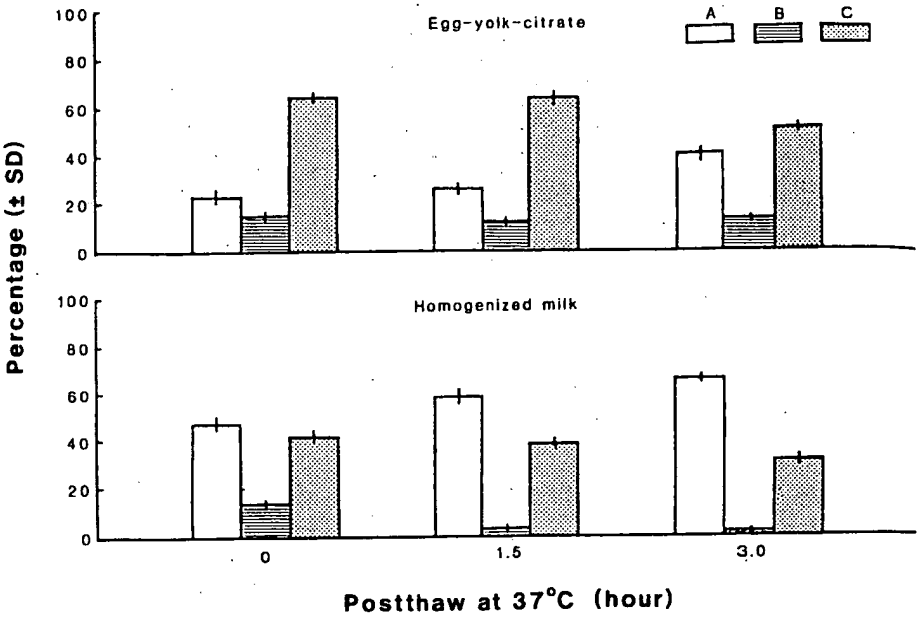

Figure 3. Flow cytometric quantification of spermatozoal population shifts occurring during incubation of thawed, cryopreserved bovine spermatozoa that had been processed and stored as paired samples in egg yolk-citrate and homogenized milk media. Population shifts were determined by analyses of populations A (open columns), B (lined columns) and C (stripled columns). Flow analyses were conducted immediately after thawing and again after 1.5 and 3.0 h at 37.5°C. The spermatozoal populations were quantified from aliquots containing spermatozoa stained with carboxyfluorescein diacetate and propidium iodide. The means represent four replications of samples from four bulls for each time interval.

No differences in correlation coefficients were noted among bulls or replications ($P>0.05$). Nearly identical patterns of change were noted for each of the extenders for the four bulls.

DISCUSSION

The spermatozoal populations in both egg yolk-citrate and milk-based media appeared identical to those previously reported for spermatozoa stored in egg yolk-citrate medium (6). Microscopic examination using epifluorescence indicated that the opacity of milk-based extenders can be readily overcome using CFDA and PI staining. The suitability of samples of cryopreserved bovine spermatozoa stored in milk extenders for flow cytometry was readily established. Discernible differences in apparent spermatozoal viability between spermatozoa frozen in homogenized milk and egg yolk-citrate based extenders were detected using dual fluorogenic staining and flow cytometry.

Although it is possible that the milk extender could have reduced the permeability of spermatozoa to CFDA and thereby decreased the estimate of the number of viable spermatozoa. Such changes would also be evident by increased numbers of PI staining spermatozoa. It should be noted that magnitude of such changes would have to be very large to have accounted for the differences noted in this study. The action of extracellular esterases originating from seminal plasma, degenerative spermatozoa and/or the extender could contribute to nonspecific staining of the surface of spermatozoa. In a previous study, however, relatively little nonspecific florescence was noted on spermatozoa that were sonicated prior to or after staining with CFDA (6). It is unlikey that nonspecific staining contributed to an overestimation of viable spermatozoa.

The importance of the possible extender differences found in this study to fertilizing capacity has not been established. The results of our study do indicate the need for more definitive studies on the effect of extender composition on spermatozoal viability and potential fertility. Other researchers (13) have reported that the extender does not affect fertility, even when minimal numbers of spermatozoa are used for insemination. The potential of this automated approach for rapid assessment of functional spermatozoal parameters, however, remains encouraging.

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Analysis of Sperm Cell Viability, Acrosomal Integrity, and Mitochondrial Function Using Flow Cytometry[1]

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ABSTRACT

A triple staining procedure was developed to evaluate bull spermatozoa using flow cytometry. Flow cytometric estimates of cell viability, measured by propidium iodide (PI) exclusion, and acrosomal integrity, measured by *Pisum sativum* agglutinin (PSA) binding acrosomal contents, were equivalent to estimates made by using standard laboratory assays. Mitochondrial function, measured by rhodamine 123 (R123) fluorescence, was depressed by the mitochondrial inhibitors rotenone (64%) or monensin (52%), establishing that mitochondrial damage can be detected. Dilauroylphosphatidylcholine (PC12) or lysophosphatidylcholine (LPC) was used to destabilize sperm membranes. When challenged with 15–30 μM PC12, selective exposure of PSA binding sites occurred without induction of PI uptake or loss of R123 staining. However, PC12 concentrations >60 μM resulted in a loss of R123 fluoresecence intensity. In contrast, >1200 μM LPC was required to expose PSA binding sites, which also resulted in PI uptake. By using flow cytometry, these three stains in combination can be used to correlate three different features simultaneously on individual spermatozoa and assay thousands of cells per sample without extensive preparation.

INTRODUCTION

Methods for evaluating semen quality prior to distribution for insemination or after experimental treatment in the laboratory are undergoing continual development in an effort to accurately estimate fertility. Unfortunately, current laboratory assays do not accurately predict fertility and are not repeatable from one study to another (Graham et al., 1980). Repeatability can only be achieved by increasing objectivity in the assays and the number of cells analyzed, while predictive capability will only be improved by measuring the correct semen parameters (which have yet to be completely determined).

A sperm cell consists of several membrane compartments (i.e. plasma membrane, acrosomal membrane, mitochondrial membrane) and cell competancy requires that each of these membrane compartments be intact. Individual laboratory assays, which evaluate these compartments singly, are not effective predictors of the fertility of a semen sample (Graham et al., 1980); however, the combination of several assays may better predict fertility. Several procedures have been used to (a) differentiate live and dead cells by the ability of an intact plasma membrane to prevent stain entry into the cell (Mayer at al., 1951; Swanson and Bearden, 1951; Hackett and Macpherson, 1965), (b) evaluate the integrity of the acrosomal membrane (Saacke and Marshall, 1968; Wells and Awa, 1970; Chacarov and Mollova, 1976; Bryan and Akruk, 1977; Cross et al., 1986; Cross and Overstreet, 1987; Cross and Meizel, 1989), or (c) evaluate both cell viability and acrosomal integrity at the same time (Talbot and Chacon, 1981; Aalseth and Saacke, 1986; Cross and Meizel, 1989; Didion et al., 1989; Ericsson et al., 1989). All these procedures require time-consuming preparation and evaluation. Consequently, when these procedures are used, the number of spermatozoa examined is usually small (<200).

Flow cytometry offers the possibility of analyzing thousands of cells in a very short time (<1 min) with precision and without the extensive preparation necessary for dried sperm smears. It has been used to correlate sperm chromatin structure with sire fertility (Evenson et al., 1980; Ballachey et al., 1987, 1988), to detect and separate sperm containing the X and Y chromosomes (Garner et al., 1983; Pinkel et al., 1985), to analyze sperm morphology (Pinkel et al., 1979), and to assess sperm cell viability (Matyus et al., 1984; Garner et al., 1986, 1988). The simultaneous analysis of cell viability and mitochondrial activity using flow cytometry has been reported (Evenson et al., 1982; Evenson and Ballachey, 1986; Auger et al., 1989) and a correlation between mitochondrial fluorescence intensity and sperm motility established (Evenson et al., 1982; Auger et al., 1989). Flow cytometric assays, however, have seldom been correlated with standard laboratory assay results.

These experiments were conducted to compare standard laboratory assays with flow cytometric assays to determine the effectiveness of flow cytometry to evaluate (1) cell viability, (2) acrosomal integrity, and (3) mitochondrial function separately and to develop a technique to analyze these characteristics simultaneously on individual cells in a sperm population.

MATERIALS AND METHODS

A series of experiments was designed to measure and compare individual sperm cell characteristics by flow cy-

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tometry and standard laboratory assays. Initial experiments tested single characteristics: (a) cell viability, (b) acrosomal integrity, and (c) mitochondrial functionality. A final experiment measured these paramaters simultaneously on individual spermatozoa. In these experiments, we define (a) viable cells as cells that exclude propidium iodide (PI) or eosin, while dead cells stain with either dye; (b) acrosome-intact cells as cells that do not bind the fluorescently labeled lectin *Pisum sativum* agglutinin (PSA) in the acrosomal region or stain with naphthol yellow and erythrosin b (Lenz et al., 1983), while acrosome-reacted cells bind PSA; and (c) functional mitochondria as those that exhibit maximal rhodamine 123 (R123) fluorescence (Johnson et al., 1980; Chen et al., 1981; Evenson et al., 1982).

Evaluation of Sperm Cell Viability

Experiments were conducted to evaluate the use of PI to determine sperm cell viability by flow cytometric analysis. To validate this technique, semen samples were assayed both by flow cytometry and by eosin/nigrosin staining of dried smears. The parity of the two assays was then determined. Samples for analysis were prepared either by killing spermatozoa by plunging them into liquid nitrogen and adding portions back to untreated cells or by aging spermatozoa for up to 48 h at 5°C.

Analysis of spermatozoa with freeze-killed cells added. Bull ejaculates were collected with an artificial vagina and processed within 1 h of collection by diluting ten times with TALP medium (Graham et al., 1986) and washing twice by centrifugation at 300 × *g* for 8 min to collect the sperm pellet. The sperm pellet was resuspended at 100 × 10^6 cells/ml and divided in two fractions. One fraction was maintained at 25°C while the cells in the other fraction were ruptured by two cycles of plunging into liquid nitrogen and thawing at 37°C. Samples for analysis were made by combining aliquots of unfrozen and frozen-thawed sperm in ratios of 1:0, 1:3, 1:1, and 3:1 (vol:vol), respectively. A 10-μl volume of PI (Sigma, St. Louis, MO) (1 mg/ml water) was added to 400 μl sperm suspension and the sample was incubated 5 min; the cells were analyzed by flow cytometry to evaluate the percentage of the dead cells (PI-positive). At the same time, subsamples were prepared for analysis using eosin/nigrosin stain (nigrosin, 5% w/v; eosin-bluish, 0.6% w/v; sodium citrate dihydrate, 3% w/v in 3% citrate) as described by Swanson et al. (1951). Dried smears were analyzed by counting 200 cells/slide using a phase microscope at 400× magnification.

Flow cytometer analysis was performed by using an Epics 753 flow cytometer (Coulter Electronics, Hialeah, FL) fitted with a beveled tip (Johnson and Pinkel, 1986). The PI was excited at 488 nm by an argon laser at 100 mW of power. Fluorescence emission was measured with a 515-nm long-pass filter and with a 610 long-pass filter for PI detection. Sperm samples were filtered immediately prior to analysis through a 40-μm nylon mesh to remove any large debris.

Regression analysis (SAS, 1985) was used to compare the flow cytometric assay with the eosin/nigrosin-staining assay.

Analysis of aged spermatozoa. Spermatozoa were collected and washed as described previously. After the second wash, cells were resuspended in 1 ml TALP, placed in a 10-ml water bath at 25°C, and cooled to 5°C in a refrigerator. Samples were removed at 0, 24, and 48 h, warmed to 25°C, diluted to 100 × 10^6 cells/ml, then stained and analyzed as described previously.

Evaluation of Acrosomal Integrity

Experiments were conducted to evaluate the use of PSA to evaluate acrosomal integrity of bull sperm using flow cytometric analysis. Semen samples were induced to undergo an acrosome reaction (AR) and assayed both by flow cytometry (using fluorescently labeled PSA) and by visual microscopic analysis of dried smears stained with naphthol yellow/erythrosin b (NY/EB) to assess the percentage of cells without an intact acrosome. To determine whether PSA binding is procedure-specific, two methods of inducing an AR were used: dilauroylphosphatidylcholine (PC12) liposomes and lysophosphatidylcholine (LPC).

Induction of the AR by PC12 liposomes. Bull ejaculates were obtained as described in the first experiment. A sperm AR was induced using PC12 as described by Graham et al. (1986). Briefly, PC12 liposomes were prepared by extrusion (Hope et al., 1985) using a 10-ml capacity Extruder (Lipex Biomenbranes, Inc., Vancouver, BC) with a 0.1-μm filter, and the liposomes were stored at −50°C until used. Sperm were washed twice and resuspended to 20 × 10^6 cells/ml. Liposomes were thawed and volumes of 0 to 120 μl were added to 200 μl sperm suspension and the final volume was brought to 400 μl with TALP. Liposomes were incubated with sperm at 39°C for 20 min, a sufficient time to induce the AR. Final PC12 concentrations ranged from 0 to 96 μM, as preliminary results indicated that this range of PC12 resulted in samples containing 5–100% AR sperm.

After incubation, 10 μl of PI (1 mg/ml in water) and 20 μl fluorescein isothiocynate (FITC)-labeled PSA (E-Y Lab. Inc., San Mateo, CA) (0.1 mg/ml in TALP) were added to the sperm samples; this gave a ratio of 0.5 μg lectin per 1 million cells.

Dried sperm smears were made at the time of flow cytometric analysis, and these were subsequently stained with NY/EB as described by Graham et al. (1986). Two hundred cells per slide were analyzed by phase microscopy to determine the percentage of cells without an intact acrosome (i.e. exhibiting an AR).

Flow cytometric analysis was performed as in Experiment 1, with the addition of a 560-nm beam-splitting filter and a 525-nm band-pass filter for FITC-PSA detection.

The sperm samples were separated into live, dead, and total populations using PI as described in the previous experiment. The forward-light-scatter gate was set (see Fig. 1a) to allow determination of the total cell population without including cell debris or aggregates. All cells accepted for analysis satisfied the forward-light-scatter gate. Live cells were PI-negative and dead cells were PI-positive. Representative histograms of fluorescence for the total sperm population, live fraction, and dead fraction are presented (Fig. 1b–d). Within the live cell population, a subpopulation of AR cells bound PSA, resulting in a sharp fluorescent peak (Fig. 1c). The dead cell population contained cells that exhibited low levels of PSA binding as well as a subpopulation that bound PSA at the same fluorescent intensity as AR live cells (Fig. 1d). Microscopic analysis of the dead cells revealed sperm with FITC labeling at the acrosomal region as well as cells with FITC labeling not specific to the acrosome. To eliminate cells exhibiting this nonacrosomal PSA binding during analyses for the AR in the total and dead cell populations, a minimum cursor position was set, with the minimum channel used for the live cells that were PSA-positive (see Fig. 1c); only those cells in the total and dead populations with PSA fluorescence above that cursor position were accepted as AR.

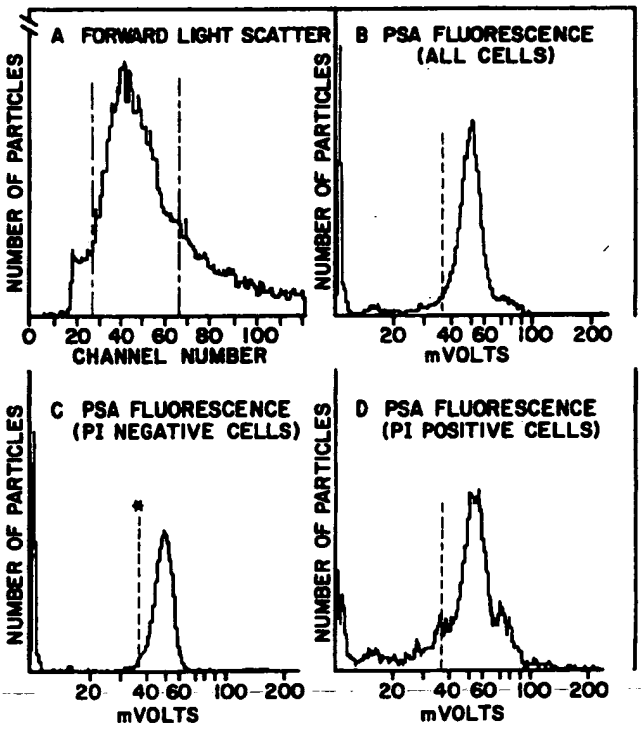

FIG. 1. Frequency histograms of bull spermatozoa evaluated by flow cytometry after treatment with 35 μM PC12 and staining with PI and fluorescently labeled PSA. (*A*) Forward-light-scatter distribution of spermatozoa. *Dashed lines* indicate thresholds used to distinguish cells (within dashed lines) from debris and cell aggregates. (*B*) PSA fluorescence intensity of the total sperm population. The *dashed line* indicates minimum fluorescence required for cell to be classified as AR. This value was determined from analysis of the live cell population. (*C*) PSA fluorescence intensity of the live cell population. The *dashed line* indicates the minimum fluorescence required for AR classification, and the *asterisk* indicates that this is the population of cells used to set the threshold for AR classification in the total and dead cell populations. (*D*) PSA fluorescence intensity of the dead (PI-positive) cell population. The *y* axis has been expanded fivefold that of the axes in *Panels B* and *C* to detail the nonspecific PSA binding below the minimum AR threshold (*dashed line*) in the dead cell population.

Regression analysis was performed on the percentages of sperm without intact acrosomes (i.e. AR sperm) for data obtained from flow cytometry and stained dried smears. Analysis of variance (SAS, 1985) was used to determine differences between the percentages of AR cells in total, live, and dead sperm populations.

Induction of the AR by LPC. A second method for inducing an AR was used to determine if this flow cytometric assay was procedure-specific. Bull sperm, washed as in the previous experiments, were treated with egg LPC (Sigma Chemical Co.) using a modified procedure of Parrish et al. (1988). Briefly, a 100-μl aliquot of LPC (5 mg/ml in methanol) was transferred to a glass tube and the methanol was removed with nitrogen gas. A volume of 500 μl TALP was added and the solution was mixed with a vortex mixer for 30 s. Several different volumes of LPC in TALP (0–45 μl) were added to 400 μl of washed sperm at 20×10^6 cells/ml, and additional TALP was added to a final volume of 500 μl. Samples were incubated 20 min at 39°C, after which they were treated with PSA and PI (at equivalent concentrations as in the previous experiments) and analyzed as were samples in the previous experiment.

Evaluation of Mitochondrial Function

The probe R123 has been shown to accumulate specifically in the mitocondria of living cells (Johnson et al., 1980; Chen et al., 1981). R123 has been used in flow cytometry to analyze mammalian cells in culture (Darzynkiewicz et al., 1982) as well as spermatozoa (Evenson et al., 1982; Evenson and Ballachey, 1986). The experiment reported here was conducted to evaluate the use of R123 to determine sperm mitochondrial function with flow cytometry. To validate this technique, semen samples were treated with several metabolic inhibitors to determine if changes in mitochondrial activity were associated with changes in R123 fluorescence intensity.

The mitochondrial inhibitors, rotenone and monensin, dissolved in ethanol, and the glycolytic inhibitor, sodium fluoride, dissolved in water, were prepared at concentrations so that, when added to sperm, inhibitor concentrations of 1×10^{-5} M, 1×10^{-4} M, and 1×10^{-3} M, respectively, would be achieved.

Bull ejaculates were collected and washed as previously described. Sperm were resuspended to 20×10^6 cells/ml in TALP, and 5 μl of inhibitor was added to each 500-μl sperm sample. These samples were incubated at 25°C for

20 min, after which 10 μl R123 (Sigma) (0.01 mg/ml in water) was added and incubated an additional 20 min. Samples were then centrifuged at 300 × *g* for 8 min to collect the sperm pellet, which was resuspended in 0.45 ml TALP and PI was added as previously described.

Flow cytometric analysis was performed using the 488-nm line of an argon laser for excitement. Filter setup included a 515-nm long-pass filter, a 590-nm dichroic beam splitter, a 525-nm band pass for green (R123) fluorescence, and a 610-nm long-pass filter for red (PI) fluorescence.

Peak fluorescence channels for each replicate were determined with the EPICS 753 MDADS computer, then were converted to voltage, which is a linear measurement, by using log-linear calibration curve. This value reflects the intensity of the signal. Treatments were analyzed by analysis of variance (SAS, 1985). Treatment differences were determined by Student-Newman-Keuls multiple-range test (SAS, 1985).

Simultaneous Evaluation of Cell Viability, Acrosomal Integrity, and Mitochondrial Function

This experiment was conducted to establish a method that could evaluate three fluorescent markers in a sperm cell simultaneously. Using R123 for mitochondrial assessment necessitated using PSA conjugated to a fluorochrome other than FITC. Phycoerythrin (PE)-conjugated PSA (Biomeda Corp., Foster City, CA), which binds sperm identically—as does FITC-conjugated PSA (data not included), was used in place of FITC-conjugated PSA.

Semen was collected and washed as previously described. Sperm were resuspended to 20 × 10^6 cells/ml and 10 μl R123 (0.01 mg/ml) was added to 400 μl sperm; samples were incubated 20 min in the dark and then centrifuged at 300 × *g* for 8 min. The supernatant was removed and the sperm were resuspended to 20 × 10^6 cells/ml. PC12 liposomes were added to 200 μl sperm and the volume was brought to 400 μl with TALP. The samples were incubated at 39°C for 15 min to induce an AR. After incubation, 10 μl PI (1 mg/ml) and 10 μl phycoerythrin conjugated PSA (0.1 mg/ml) were added to the spermatozoa 5 min prior to analysis on the flow cytometer.

Flow cytometric analysis was performed using an argon laser at 488 nm (100 mWatt) for excitation. Filter set up included a 515 nm long pass filter with a 457–505 nm laser blocker; a 550 nm dichroic beam splitter and a combination of a 525 nm band pass with a 560 nm short pass filter for R123 (photomultiplier tube high voltage set at 1100); a 590 nm dichroic beam splitter, a combination of a 580 nm band pass with a 570 nm long pass filter to detect PE (photomultiplier tube high voltage set at 1300), and the combination a 610 nm long pass filter with a 630 nm long pass filter for PI (photomultiplier tube high voltage set at 900). With these settings, immuno check beads (Coulter Electronics, lot #5171) had fluorescent intensities corresponding to channel numbers 194, 248 and 217 for the R123, PE and PI photomultiplier tubes, respectively. The intensity of fluorescein labelled beads having 1.8 × 10^5 equivalent soluble molecules/bead (Flow Cyometry Standards Corp., Research Triangle Park, NC, lot #060188) were also determined for the R123 photomultiplier tube, having an intensity corresponding to channel 43.

RESULTS

Evaluation of Sperm Viability

A comparison of percentages of dead (stained) cells analyzed by eosin/nigrosin staining and by flow cytometry when frozen—killed cells were added to samples is presented (Fig. 2). The 95% confidence interval of the regression line for the data encompassed the line representing a 1:1 correlation of the two techniques, indicating that equivalent percentages of dead cells were measured by the two procedures.

Comparing eosin/nigrosin staining with flow cytometry of aged cells yielded similar results. Data from six replicates produced PI-stained cells ranging from 7% to 41%, and the regression line $Y = 4.8 + 0.8X$, where Y = % dead estimated by microscopy and X = % dead estimated by flow cytometry ($r = 0.778$). The 95% confidence interval of this regression line encompassed the line representing a 1:1 correlation of the two techniques, as did the previous experiment.

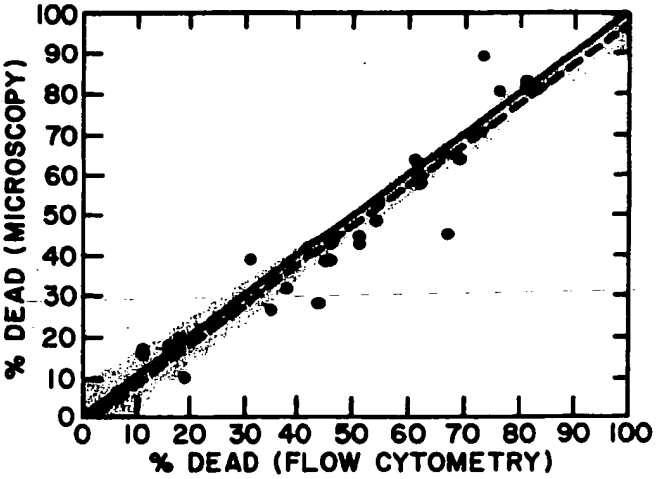

FIG. 2. Comparison of the percentage of stained (dead) cells in sperm samples by microscopic analysis using eosin/nigrosin-stained dried preparations and flow cytometric analysis using PI-stained cells. The percentage of dead cells determined on each sample using both methods (●), the regression line defined by the data (·····) and, the line of theoretical equivalence of the two assays (——) are presented. The *shaded area* represents the 95% confidence interval of the data (n = 7).

Microscopic analysis of PI-treated samples also revealed that motile cells never took up this stain.

Evaluation of Acrosomal Integrity: PC12

After treatment with PC12 liposomes, sperm exhibiting an AR could be distinguished as live or dead by flow cytometry. This was not possible with the NY/EB stain. Therefore, the percentages of the total cell population exhibiting an AR (stained with PSA) as determined by flow cytometry and by the NY/EB stain are shown in Figure 3A. Again, the 95% confidence interval of the regression line encompassed the line of a 1:1 correlation of the two procedures, indicating that the percentages of cells exhibiting an AR as determined by these two procedures were similar.

When the flow cytometer analyses were conducted to accept all stained cells (including those with low fluorescence; see Fig. 1) as AR, the flow cytometer detected approximately 6% more AR cells than would be expected if the two types of analysis were equivalent (see Fig. 3A). The 95% confidence interval of this regression line failed to encompass the line that indicates equal evaluation of the percentages of AR cells using these two procedures (data not shown).

The percentage of cells exhibiting an AR when sperm were treated with various concentrations of PC12 are shown for both the total cell population and the live population (Fig. 4A). The percentage AR cells in the live population was lower than that of the total population when fewer than

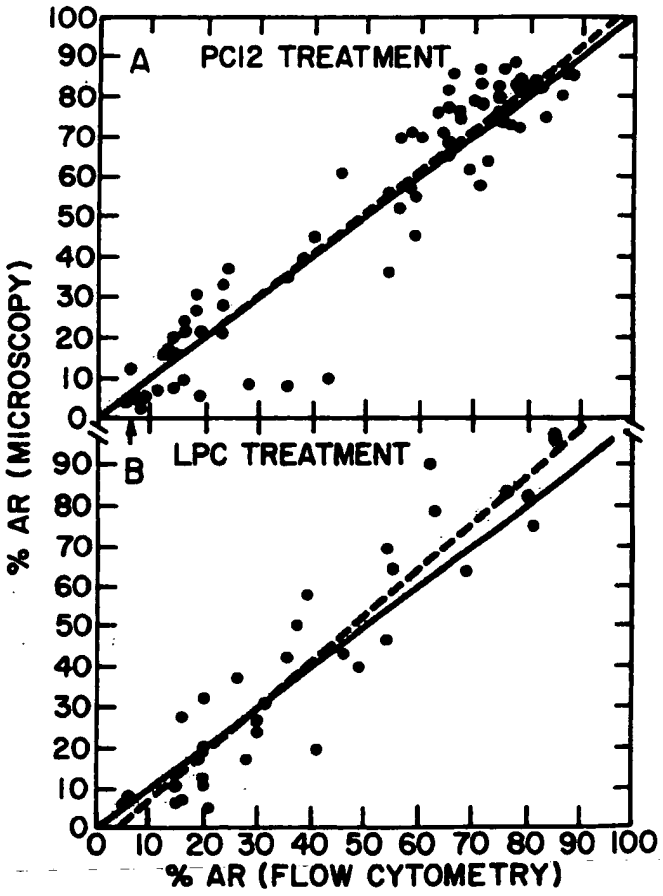

FIG. 3. Comparison of the percentage of AR cells in sperm samples measured by microscopy, using NY/EB-stained dried preparations, and flow cytometry, using PSA-labeled cells, with a lower gate set to eliminate nonspecific PSA binding. The percent AR in each sample using both assays (•), the regression line defined by the data (·····), and the line of theoretical equivalence of the two assays (——) are presented. The *shaded area* represents the 95% confidence interval of the data. (*A*) Spermatozoa were treated with PC12 liposomes to induce an AR (n = 12). When flow cytometric analyses included all cells stained with PSA, including those with PSA fluorescence below the AR threshold, comparisons with microscopic analyses produced the equation Y = 6 + 1X; this regression line runs parallel to the line of theoretical equivalence but intersects the *X* axis at the *arrow*, not at the origin. (*B*) Spermatozoa were treated with LPC to induce an AR (n = 5).

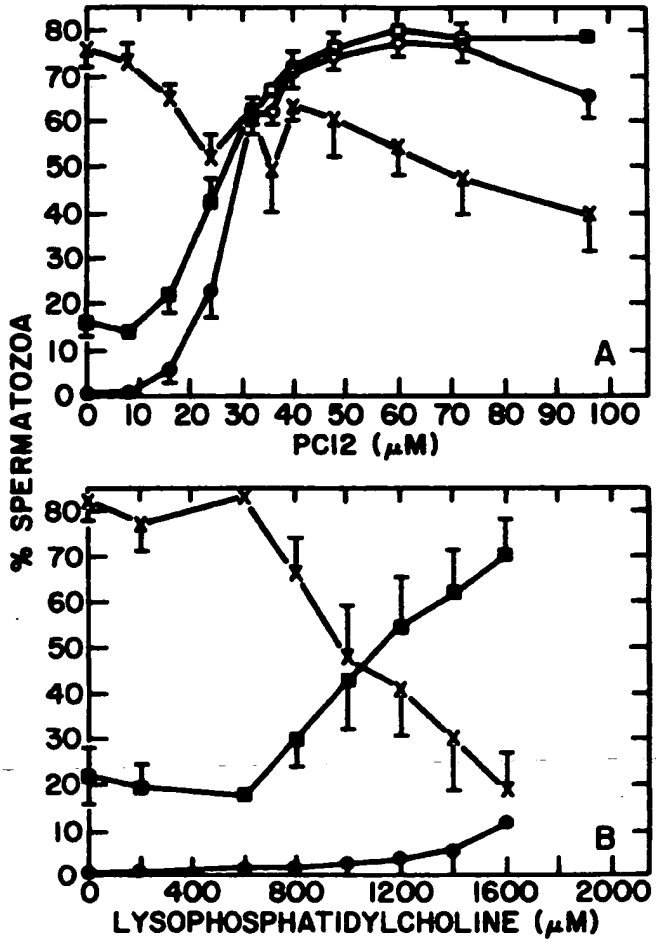

FIG. 4. The percentage of live (PI-negative) cells (X) and the percentage of cells in the total sperm population (□) and the live (PI-negative) cell population (○) exhibiting an AR when assayed for PSA labeling using flow cytometry after treatment to induce an AR. *Open symbols* (○, □) indicate that the percentage of AR cells in the total and live populations were not different; *solid symbols* (•, ■) indicate a difference at $p < 0.05$. (*A*) Spermatozoa were treated with PC12 liposomes to induce an AR (n = 12). (*B*) Spermatozoa were treated with LPC to induce an AR (n = 5).

50% of the total cells exhibited an AR. Dead cells constituted between 25% and 60% of the total cell population at any PC12 concentration (Fig. 4A); this subpopulation averaged 68% AR at these lipid concentrations, ranging from a minimum of 49% to a maximum of 82%.

Evaluation of Acrosomal Integrity: LPC

After treatment with LPC, the percentage of the total cell population stained with PSA detected with flow cytometry was compared with the percentage of cells detected as AR with the NY/EB stain (Fig. 3B). The 95% confidence interval of this regression line encompassed the 1:1 ratio of these two analysis procedures.

The percentage of the cells that were alive as well as the percentage of cells exhibiting an AR in both the total cell and live cell populations are presented in Figure 4B. The percentage of live cells decreased while the percentage of AR cells increased at similar, although opposite, rates. The percentage of live cells exhibiting an AR was less than the percentage of the total cell population exhibiting an AR at every LPC concentration. Although the percentage of live cells exhibiting an AR also increased with LPC concentration, it did so at a lower rate.

Between 60 and 70% of the dead cells were AR at every LPC concentration. The percentage of dead cells, however, increased from less than 20% at 0 μM LPC to over 80% of the total cell population at 1 600 μM LPC.

Evaluation of Mitochondrial Function

The addition of 1% ethanol (solvent for monensin and rotenone) to sperm did not affect either the percentage of dead cells in the sample or the intensity of R123 fluorescence (Table 1). The glycolytic inhibitor (NaF), also in the presence of ethanol, had no effect on either the percentage of dead cells or on R123 fluorescence. The addition of the mitochondrial inhibitors rotenone and monensin decreased the fluorescence intensity of R123 in the live cells, but did not change the percentage of dead cells in the population. The dead population contained few cells with any R123 fluorescence, and the few that exhibited any R123 fluorescence exhibited only low levels of intensity.

TABLE 1. The intensity of rhodamine 123 fluorescence in spermatozoa and the percentage of dead cells when bull sperm were treated with various metabolic inhibitors (n = 6).

Treatment	Percent of voltage for control-2	Percent dead
Control-1 (no addition)	108[a]	18[a]
Control-2 (ethanol addition)	100[a]	18[a]
NaF + ethanol	101[a]	17[a]
Rotenone + ethanol	64[b]	20[a]
Monensin + ethanol	52[b]	19[a]
SEM	6	2

[ab]Denote differences within columns at $p < 0.05$.

Simultaneous Evaluation of Cell Viability, Acrosomal Integrity, and Mitochondrial Function

Spermatozoa were analyzed for PI, bound PSA, and R123 fluorescence (Fig. 5). Spermatozoa positive for PI (dead) were identified and eliminated from further analysis; PI-negative cells were further analyzed for PSA binding (AR) and R123 (mitochondrial function). After treatment with PC12 liposomes, subpopulations of sperm were detected that exhibited AR and functional mitochondria (Fig. 6). With this procedure, changes in the percentage of dead sperm and the status of the acrosome and mitochondria of the live sperm were detected when spermatozoa were treated with increasing levels of PC12 liposomes (Fig. 7). In control samples, most spermatozoa contained active mitochondria, but few live cells exhibited an AR. The addition of low concentrations of PC12 liposomes induced an AR in spermatozoa without reducing R123 fluorescence. Higher concentrations of PC12 (≥60 μM) reduced mitochondrial function in live AR spermatozoa.

DISCUSSION

Established microscopic procedures for evaluating populations of sperm cells are hindered by involved preparation and time-consuming analysis; consequently, sample size is small. Flow cytometry offers the possibility of objectively

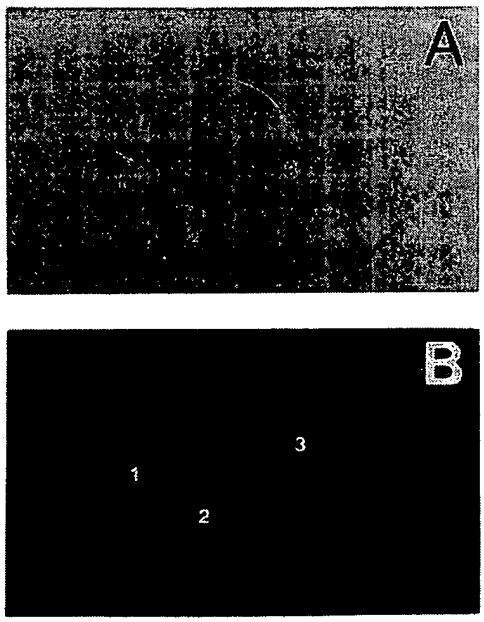

FIG. 6. Paired bright-field (*A*) and epifluorescent (*B*) photomicrographs of PC12-treated bull sperm stained with PI, PE-PSA, and R123. Spermatozoon 1 demonstrates a live cell with a moving flagellum. This cell is PI-negative, PSA staining over the acrosome reveals an AR cell, and R123 staining of the mitochondria is present. Spermatozoon 2, also alive with a moving flagellum, is PI-negative and exhibits R123 staining of the mitochondria but has an intact acrosome (PSA-negative). Spermatozoon 3 exhibits no flagellular movement, is PI-positive and has no R123 fluorescence.

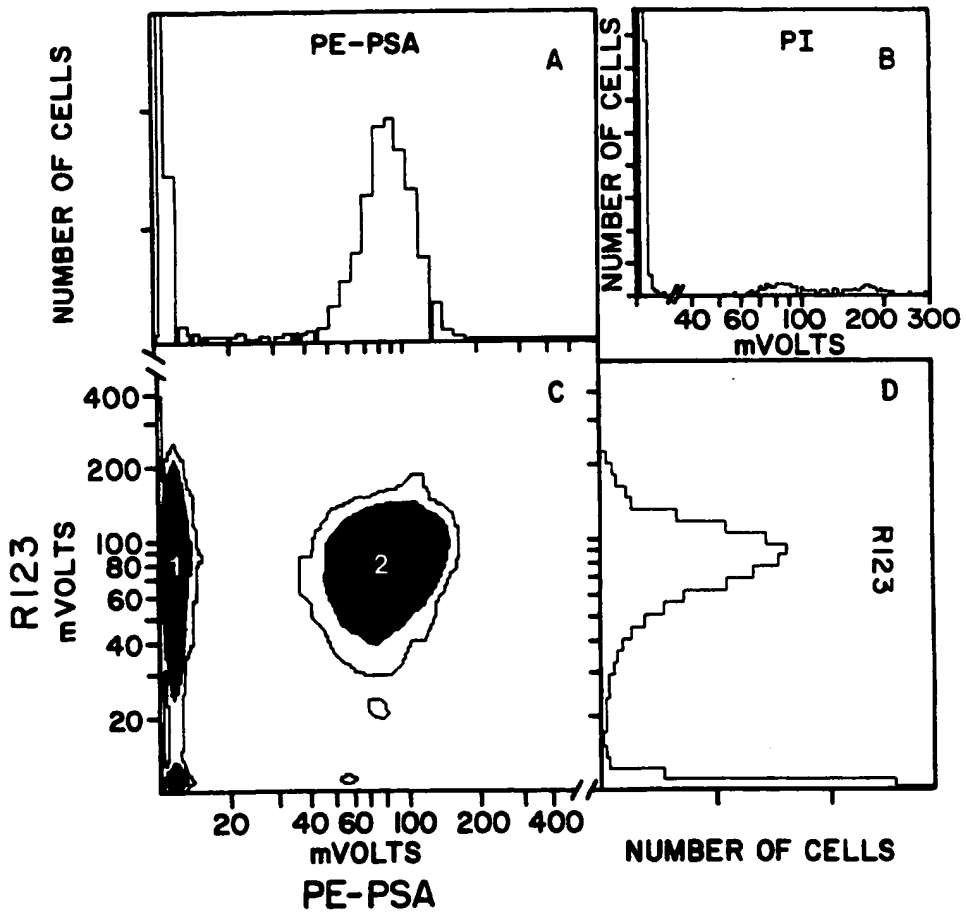

FIG. 6. Example of the analysis of spermatozoa stained with PI, R123, and PE-PSA after sperm were treated with 15 μM PC12. Procedures used are described in the *text*. (*A*) Histogram of PE-PSA fluorescence bound to sperm that excluded PI. (*B*) Histogram of PI fluorescence intensity of sperm cells. (*C*) Scatter-plot of cells that excluded PI showing two subpopulations of sperm; both exhibit high R123 fluorescence, one of which has no PE-PSA fluorescence (*1*) and one of which exhibits high PE-PSA fluorescence (*2*), indicating that these are AR cells. (*D*) Histogram of R123 fluorescence intensity of sperm cells that excluded PI.

measuring thousands of cells for multiple characteristics in a short time with minimim preparation. The present studies establish a correlation between the flow cytometric analyses of several sperm membrane compartments and the established laboratory assays for those same compartments and establishes a protocol to evaluate them simultaneously.

PI cannot pass through an intact plasma membrane, but passes into and stains the nuclei of degenerated spermatozoa (Garner et al., 1986). Assays using PI and assays using eosin/nigrosin stains for intact plasma membranes produced nearly equivalent results (Fig. 2), indicating that PI is an accurate supravital stain for sperm analyzed by flow cytometry. The correlation for the two assays was not perfect, probably due to differences in the size of the populations assayed (200 cells for eosin/nigrosin and 10 000 cells for flow cytometry). Garner et al. (1986) showed a significant negative correlation with the population of cells stained with PI and the percentage of motile sperm, which is reasonable since dead sperm are not motile. A significant negative correlation has also been found between PI-stained sperm and sperm containing an intact acrosome (Garner et al., 1986). Matyus et al. (1984) reported that the percentages of dead sperm in semen samples from four bulls, as measured by the combination of fluorescein diacetate and PI, after incubating sperm at 55°C for 15 min, is correlated with sire fertility. Ethidium bromide (Evenson et al., 1982) and hydroethidine (Ericsson et al., 1989) have also been used as supravital stains to stain dead cells for flow cytometric analysis.

Some sperm stain only partially with eosin B (Swanson and Bearden, 1951). Similarly, populations of slightly stained cells have been detected on the flow cytometer with PI (Garner et al., 1986) and hydroethidine (Ericsson et al., 1989). These populations were detected in our analyses by using dried smears and the flow cytometer. We classified such cells as dead (stained), since they probably represented dying cells and would not contribute to the viable cell population.

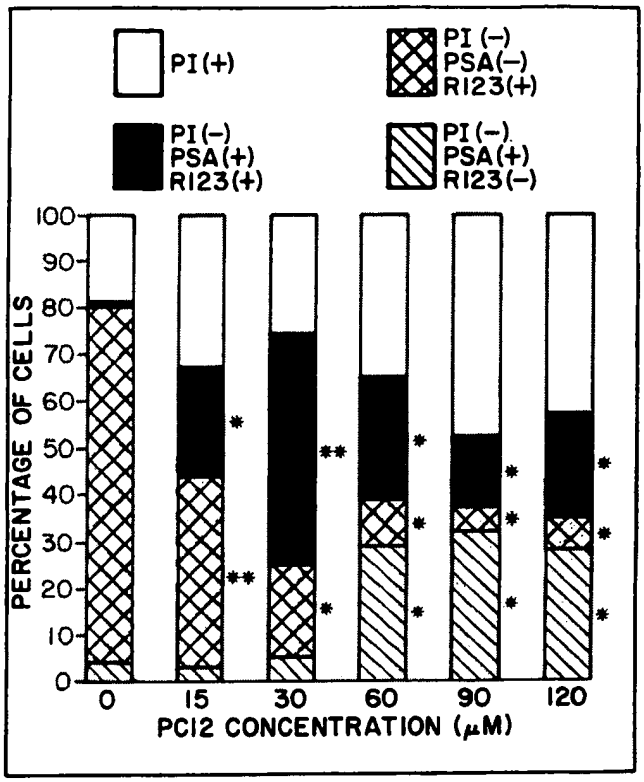

FIG. 7. The percentage of PC12-treated cells that were classified as dead (PI-positive); viable with maximally functioning mitochondria and an AR (PI-negative, R123-positive and PSA-positive); viable with intact acrosomes and maximally functioning mitochondria (PI- and PSA-negative, R123-positive); and viable exhibiting an AR but with suboptimal mitochondrial function (PI- and R123-negative, PSA-positive). Within each classification of sperm (described above), different numbers of asterisks (*, **) denote differences between PC12 concentrations at $p < 0.05$ ($n = 4$).

Some sperm exhibit low levels of PSA binding (Fig. 1). When sperm containing low (possibly nonspecific) levels of PSA binding were eliminated from the AR population, the two assays (flow cytometry and NY/EB) for percentage of intact acrosomes were similar, indicating that fluorescently labeled PSA can be used in flow cytometry to assess the percentage of cells with (or without) intact acrosomes (Fig. 3). When sperm exhibiting low fluorescence were included in the AR population, flow cytometry tended to overestimate the percentage of AR cells by approximately 6% compared with the NY/EB assay. The results obtained in these experiments indicate that flow cytometry can be used to analyze the percentage of cells with intact acrosomes when an AR is induced by various methods (PC12 and LPC).

PSA has been used previously for microscopic assay of acrosomes in ethanol-fixed human sperm cells (Cross et al., 1986). Ethanol fixation of bull sperm prior to addition of PSA for assay by flow cytometry had no correlation with dried sperm smears stained with NY/EB (data not presented), although acrosomes could be visualized by fluorescent microscopy. Ethanol fixation would also mandate an additional washing of the cells if cell viability were examined concurrently with AR and would prohibit mitochondrial analysis. It is hypothesized that without fixation AR cells retain enough of the acrosomal contents on the inner acrosomal membrane to be detected on the flow cytometer. These cells can also be differentiated by using microscopy.

The percentage of AR cells increased with the concentration of PC12 or LPC added to the samples (Fig. 4). Similar results have been reported previously for PC12 liposomes (Graham et al., 1986) and for LPC (Parrish et al., 1988). In PC12-treated samples, the percentage of AR cells in the total cell population was higher than that of the live cell population when less than 50% of the total population was AR. This difference was due to the number of cells in the dead population that lacked intact acrosomes. The dead cell population consistently contained 50–80% of the cells without intact acrosomes. In LPC-treated samples, the percentage of AR cells in the total cell population was higher than that of the live population at all LPC concentrations. Similar trends in percentage of AR cells and percentage of live cells are seen in these studies and studies reporting the percentage of AR and percentage of motile cells after LPC treatment (Parrish et al., 1988).

These two methods of inducing the AR produced very different effects on the sperm. LPC had a toxic effect on spermatozoa at concentrations that induced the AR; at LPC concentrations resulting in 70% of the total population exhibiting an AR, only 20% of the cells were alive, and only 12% of the live population exhibited an AR. In contrast, when 70% of the total population of PC12-treated cells exhibited an AR, 64% of the cells were alive, and of the live cells, 70% were AR. A similar trend was reported by Parrish et al. (1988) for spermatozoa treated with LPC alone and spermatozoa treated with heparin followed by an LPC challenge.

In extrapolating data to other systems, i.e., systems attempting to promote an AR in vitro or systems assaying the percentage of intact acrosomes for assessment of semen quality, conventional analyses may significantly overestimate the percentage of AR cells in the viable population. Determination of acrosomal integrity of the live cell population may provide more accurate predictive measurements of fertility than those currently used.

Analysis by flow cytometry does not discern specific acrosomal abnormalities that can be detected by differential interference contrast microscopy (Saacke and White, 1972). These analyses have been correlated with sire fertility, but similar correlations with flow cytometric analyses have not been done.

The fluorescent stain R123 was accumulated and retained most intensively in fully functional mitochondria (Table 1). Mitochondria from dead cells failed to accumu-

late or retain R123 after washing. Sperm treated with sodium fluoride, an inhibitor of glycolysis, had equivalent R123 fluorescence as control sperm. Mitochondria in sperm treated with rotenone or monensin (mitochondrial inhibitors) were able to retain only about half the fluorescence seen in fully functional mitochondria. This probe, therefore, can be used to detect functional mitochondria in sperm cells. Although motility was not evaluated in this study, previous work has demonstrated a correlation between R123 fluorescence and sperm motility (Evenson et al., 1982; Auger et al., 1989).

Previous studies have used two fluorescent probes for analyzing sperm cells (Evenson et al., 1982; Matyus et al., 1984; Evenson and Ballachey, 1986; Garner et al., 1986; Auger et al., 1989; Ericsson et al., 1989). We have demonstrated that by adding three probes to sperm cells, spermatozoa could be analyzed for cell viability, acrosomal integrity, and mitochondrial function simultaneously (Fig. 6 and 7). These studies demonstrate that at least three sperm compartments can be analyzed by flow cytometry. Although different flow cytometer filter combinations were used in each experiment, the filter system used for the three stain combinations can be used for any single or double-staining combination desired. In addition, these studies demonstrate the pharmacological manipulation of a sperm suspension. Contemporary knowledge of the requirements for sperm/egg fusion in in vitro systems would argue for a treatment that would result in the maximum number of sperm which are viable, have functional mitochondria, and exhibit an AR (Fig. 7). Too little treatment would result in cells with functional mitochondria but lacking an AR and too much treatment would result in AR cells with little mitochondrial function. The titration of any pharmacological agent to induce a desired effect should identify a concentration of the agent that produces a maximum effect. Analysis of other cell compartments or surface features can be done in similar fashion with an appropriate probe attached to a fluorescent marker. The power of this technique is that multiple characteristics, previously estimated on cell populations, can now be measured on individual cells. The advantage to using flow cytometry is that it can objectively evaluate thousands of cells per sample, it requires little preparation, and the analysis can be completed in very little time. Such an assay should prove very useful in the routine analysis of semen samples.

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Comparison of Motility and Flow Cytometric Assessments of Seminal Quality in Fresh, 24-Hour Extended and Cryopreserved Human Spermatozoa

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ABSTRACT: Functional differences among fresh 24-hour extended and cryopreserved human spermatozoa were assessed using both computer-assisted semen analysis (CASA) and flow cytometry. The objective was to determine if there were interrelationships among various qualitative parameters of the fresh and treated samples when assessed by these two automated methods. Fertile donor specimens ($n = 15$) were split and examined for sperm motility and curvilinear velocity using CASA within 1 hour postejaculation, after 24 hours in TEST–yolk buffer at 5° C and after cryopreservation in TEST–yolk–glycerol medium. Flow cytometric analyses were performed on 24-hour extended and cryopreserved (CP) samples after fluorescent staining with rhodamine 123 to quantify mitochondrial function and carboxydimethyl fluorescein diacetate and propidium iodide to assess plasma membrane integrity. The percentages of spermatozoa with functional mitochondria and intact membranes along with the proportion of dead cells were identified and quantified by flow cytometry. Quadrant analyses of these data were used to determine the relative red and green fluorescent intensities. The initial sperm motility was correlated to the motility observed for the 24-hour stored and the CP samples. The sperm velocity of both the initial and the 24-hour extended samples was correlated to the velocity of CP samples. As for the comparison of the two automated methods for assessing seminal quality, the only sperm motion parameter that was correlated with a sperm population identified by flow cytometry (quadrant 4) was the curvilinear velocity of the sperm after 24 hours storage ($r = 0.69$) and after cryopreservation ($r = 0.74$). The present findings indicate that additional research is needed to determine if prefreeze analyses of donor sperm could be useful in predicting the post-thaw integrity of CP samples and, thereby, be useful in screening potential semen donors.

Key words: Male germ cells, sperm function, fluorescence, sperm motion, sperm membranes.

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Automated semen evaluation techniques have been developed to attain more objective assessments of sperm cell quality. These automated systems include computer-assisted semen analyses (CASA) and flow cytometry. The CASA systems provide objective analyses of sperm motion parameters (Katz and Overstreet, 1981; Mortimer et al, 1988; Amann, 1989; Davis et al, 1992a,b). The flow cytometric approach, an automated technique that quantifies the relative staining intensity of several thousand sperm labeled with organelle-specific fluorescent stains in a few minutes, can provide information on several sperm characteristics including mitochondrial function, plasma membrane integrity, and cell viability (Evenson et al, 1982; Garner et al, 1986; Auger et al, 1989; Graham et al, 1990; Garner et al, 1992). Both of these analytical systems have been used for seminal quality determinations, but little comparative information is available especially for both fresh and cryopreserved sperm samples.

The utility of preliminary assessments of fresh human donor semen quality for estimating the potential fertility of cryopreserved samples from that particular donor has not been adequately examined. In many instances, a donor may have superior quality sperm in fresh semen, yet have a relatively low fertility rate when samples are cryopreserved. This problem becomes a central focus because the potential prevalence of human immunodeficiency virus (HIV) and the hepatitis viruses in the human population restrict the use of fresh donor semen for assisted reproductive techniques (ART) (Allen et al, 1985; Ball, 1986; New Guidelines for the Use of Semen Donor Insemination, 1990). Donor screening includes multiple tests for sexually transmitted and contagious blood-borne diseases, followed by semen cryopreservation and quarantine for 6 months. Before such cryopreserved samples can be used, the donors must be retested (New Guidelines for

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EXHIBIT G

the Use of Semen Donor Insemination, 1990). This makes cryopreservation a necessary component of any ART program.

The decreased fertility resulting from the use of cryopreserved spermatozoa not only increases the cost per pregnancy for infertility centers using donor semen but ultimately costs the patients (Ackerman and Behrman, 1975; Keel and Webster, 1989; Barratt et al, 1990). Thus, an important goal in cryopreservation programs is the ability to predict accurately the quality of cryopreserved samples of human sperm. The magnitude of decreased fertility rate incurred when using cryopreserved samples varies greatly among donors. The objective of the research reported herein was to develop a rapid and sensitive flow cytometric method for assessing sperm quality in both fresh and cryopreserved samples and to examine the interrelationships among the flow cytometrically identified sperm populations and certain sperm motion parameters as quantified by CASA.

Materials and Methods

Materials

Rhodamine 123 (R123) was obtained from Eastman Kodak Co. (Rochester, New York) and carboxydimethyl fluorescein diacetate (CDMFDA) and propidium iodide (PI) from Molecular Probes (Eugene, Oregon). The solvent, dimethylsulfoxide (DMSO), and the buffering medium, Tyrodes salt solution, were obtained from Sigma Chemical Co. (St. Louis, Missouri). The TES (*N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) and Tris (Tris[hydroxymethyl]aminomethane) were obtained from Irvine Scientific (Santa Ana, California) as TEST–yolk buffer (TYB), refrigeration medium, and TEST–yolk buffer with 12% glycerol (TYB-CP) cryopreservation medium. The solution of R123 (52.6 mM) was prepared by adding 5 mg to 25 ml of DMSO. Other reagents used were 2.0 mM CDMFDA in DMSO and 3 mM PI in Tyrodes solution (2 mg/ml).

Donor Screening Criteria

Donors were screened and assessed according to World Health Organization criteria (WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction, 1987). The semen volume, concentration, morphology, white blood cell concentration, progressive motility, and thawed cryopreserved progressive motility required by WHO are 2–5 cc, 80 million/cc, ≥70% normal, <8–10 per high-power field, ≥60%, and ≥40%, respectively. Criteria for rejection occurred if any of the above values were below/exceeded these limitations. All donors were screened according to the AFS time requirements for AIDS, VDRL, hepatitis, mycoplasma, *Chlamydia*, microbial culture and sensitivity, cytomegalovirus, and gonorrhea. Genetic screening was done on all prospective donors for cystic fibrosis. Genetic screening was done for sickle cell anemia and Tay-Sachs disease when appropriate. Drug toxicology screenings also were carried out.

Samples

Fifteen human donor specimens were obtained from proven fertile donors in the Humana–Michael Reese Hospital sperm bank program in Chicago, Illinois. Each specimen was brought to the Andrology Laboratory within 1 hour following ejaculation, and, after thorough mixing, duplicate aliquots were immediately examined for sperm motility using CASA (Cell Soft, Series 3000, New York). A portion of each specimen was diluted with TYB (Vaught et al, 1987) at 37°C and slow cooled by placing the containers in a 400-ml beaker of water (22 ± 2°C), which was subsequently placed in storage at 5°C. An aliquot of TYB precooled samples was packaged at 5°C and sent to Reno, Nevada for flow cytometric analyses. The TYB-stored samples were analyzed approximately 24 hours later, after being slowly warmed to 37°C, for both the CASA and flow cytometric analyses. Another aliquot of the fresh specimens as extended in TYB-CP cryoprotectant and slow cooled as described above. After a 2-hour cooling period, 1-ml aliquots of the specimens were transferred to precooled NUNC cryovials, placed in a cooling rack, and positioned in the neck of a liquid nitrogen storage tank for nitrogen vapor freezing. After 1 hour, the cryovials containing the extended semen were plunged into the liquid nitrogen (−196°C) and stored for a minimum of 48 hours before being thawed and analyzed by CASA and flow cytometry.

Sperm Motion Assessments

An Olympus CH-S microscope (Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a phase-contrast condenser and a 10× achromatic S-plan objective, 6.7× photo eyepiece and a high-resolution videocamera (Panasonic WV-1410, Matsushita Electric Industries, Secaucus, New Jersey) was used to assess the initial 24-hour extended and post-thaw motility parameters. The video signal was fed to a videorecorder, and the analyses were done in real time. The video image was digitized, and an image was directed to a microcomputer (NEC Power Mate, Wood Dale, Illinois), equipped with the proprietary hardware and software (CellSoft) to provide frame-by-frame analyses of sperm tracks (sampling frequency—30 frames/second). The CellSoft Module (Series 3000) was used for all analyses. Five microliters of each sample were placed on a Makler counting chamber (depth—20 μm; Irvine Scientific, Santa Ana, California) and assessed using CASA. Spermatozoa were described as motile if they met the minimum criteria for cell movement (i.e., maximum velocity = 200 μm/second, threshold velocity 8 μm/second, minimum sampling velocity (V_{CL}) 10 units, cell size 5–25 pixels; Mack et al, 1988). The sensitivity threshold was adjusted, and a minimum of 200 sperm cells was analyzed for each of two replicates. Only those cells meeting minimum tracking requirements were rated. The spermatozoa were assessed for the percentage of motile cells (Mot-1, Mot-2, and Mot-3) and for curvilinear velocity (Vel-1, Vel-2, and Vel-3). These were the only motion parameters assessed because they have been shown to have a smaller within-sample variation (Katz and Davis, 1987).

Flow Cytometric Analyses

Flow cytometry was carried out in triplicate on aliquots of spermatozoa that had been stored at 5°C for 24 hours and on identical thawed cryopreserved samples. Both the 24-hour extended and

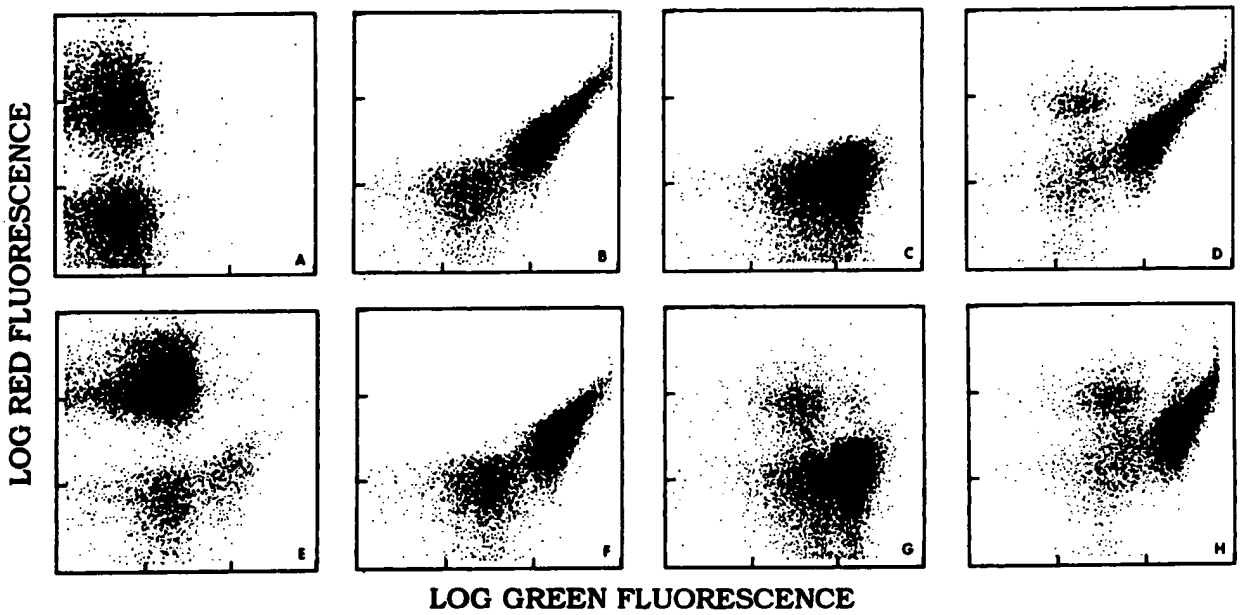

FIG. 1. Dot plots of human sperm that had been thawed and pooled from two of the donors before being stained and then analyzed using flow cytometry. The sperm cells were fluorescently stained before simultaneous measurement of both green (FL1) and red (FL2) fluorescence using a FACS analyzer. Portions of the thawed, pooled, cryopreserved sample of sperm cells were stained with propidium iodide (PI) (A); carboxydimethyl-fluorescein diacetate (CDMFDA) (B); rhodamine 123 (R123) (C); CDMFDA and PI (D); CDMFDA, R123, and PI after treating with digitonin to kill most of the sperm (E); CDMFDA and R123 (F); R123 and PI (G); and CDMFDA, R123, and PI (H). Note that two populations are evident for the individual stains and that the combination of CDMFDA and R123 (F) only exhibits two populations. This combination of green stains is additive within individual sperm cells rather than identifying an additional green population. The combination of CDMFDA and R123 tended to be more definitive (F) than when either stain was used alone (B and C). The third stain, PI, identified the dead sperm (A, D, E, G, and H; upper population), whereas media debris (egg yolk granules, etc.) and cellular debris (cytoplasmic droplets, etc.) is illustrated in the lower left corner of the dot plots. The presence of the green stains shifted the main PI population to the right due to the presence of free green dye and to some nonspecific staining on cell surfaces (compare A and E). The fluorescence distribution of 10,000 sperm was measured with an instrument equipped with an FITC/phycoerythrin dichroic filter emission set for simultaneous assessment of both green and red fluorescence for each cell. The green and red fluorescence emissions were separated optically so that they could be measured by separate photomultiplier tubes and then integrated using the standard Consort 30 Revision D software of the FACS analyzer.

the thawed cryopreserved samples were diluted 1:3 with Tyrodes solution at 37°C. Each sample was then stained with 0.8 μl R123 and incubated at 37°C for 90 minutes prior to analysis. Fifteen minutes prior to analysis, or 75 minutes after the R123 addition, the samples were further stained with 0.8 μl CDMFDA and 6 μl PI. Dot plots of the staining patterns for the individual stains (Fig. 1A–C) show that two populations are evident for each stain. Combinations of two stains identified three populations (Fig. 1D–F) with the exception of the combination of CDMFDA and R123 (Fig. 1F). This combination was additive and tended to provide a more definitive separation of the two populations (compare Fig. 1B,C with F). Treatment of the sperm with digitonin caused most of the sperm to die and stain with PI (Fig. 1E). When all three stains were combined, three populations were readily identifiable (Fig. 1D).

The samples were stained at 2-minute intervals and then incubated for 1.5 hours before being sequentially analyzed at 2-minute intervals. A total of 10,000 fluorogenic cells were cytometrically assessed for each sample. Electronic (Coulter) volume (VOL), right angle light scatter (SSC), and two fluorescence parameters (FL1 and FL2) were measured simultaneously as the individual sperm cells passed through a FACS Analyzer (Becton Dickinson Immunocytometry Systems, Mountain View, California) fitted with a fluorescein isothiocyanate (FITC) and phycoerythrin dichroic filter set. Excitation filters were an LP 400 long pass and DF 485/22 band pass. A DM-560 dichroic mirror was used to separate the fluorescent signals. The FL1 photomultiplier tube (PMT) was used to collect the 530-nm light that passed through a DF530/30 bandpass filter. The FL2 PMT was used to collect the 570-nm light that passed through two LP 570 filters. Electronic compensation was utilized (FL1 0.2 and FL2 1.9) to minimize the crossover of green fluorescence into the PMT quantifying red fluorescence. All data were collected with 3-decade logarithmic amplification. Samples were measured at a rate of 200–600 cells/second. Data were collected with the gates set to eliminate aggregates and particles from analyses. The CONSORT 30, a data analysis system utilizing a Hewlett-Packard 200 series computer with C30 version D software, was used for data collection. Four parameter data on red fluorescence, green fluorescence, Coulter volume, and light scatter were collected in list mode and examined using quadrant analyses.

The percentage of dead spermatozoa, as identified by PI staining (high red), were localized and quantified as quadrant 1 (Q_1; Fig. 2B) while the percentage of spermatozoa with functional membranes and mitochondria, as identified by CDMFDA and R123 staining (high green plus some crossover staining into the red channels), respectively, were localized and quantified as quadrant 2 (Q_2; Fig. 2B). The FITC/phycoerythrin filter system allows crossover of green fluorescence into the PMT quantifying red fluorescence. This crossover necessitates the use of electronic

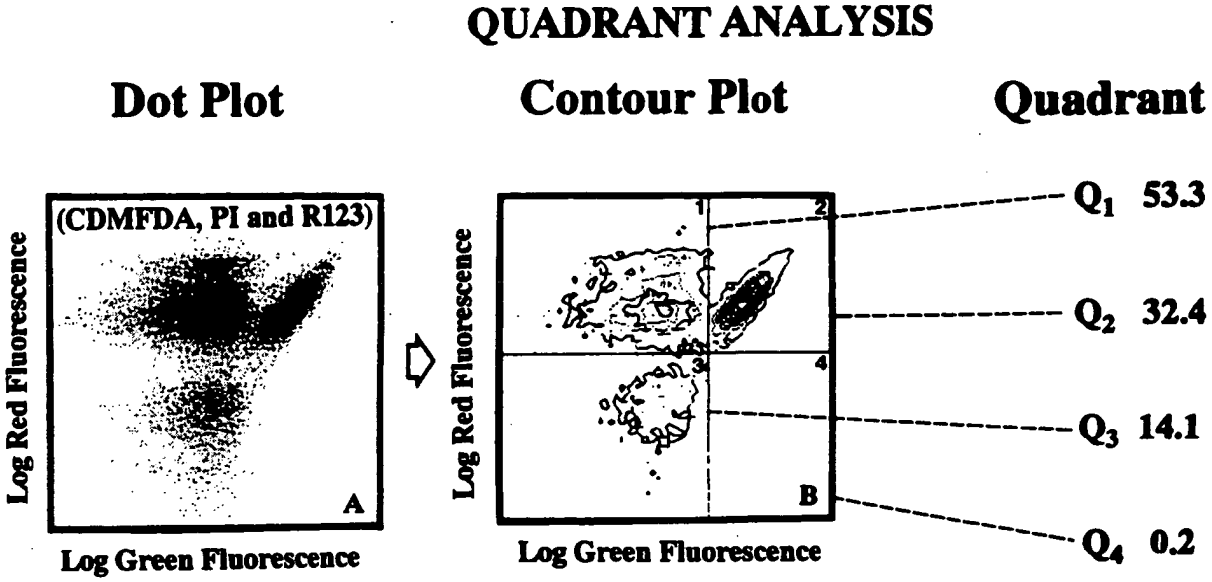

FIG. 2. Dot plot (A) showing the populations of sperm from donor #13 stained with CDMFDA, PI, and R123 and analyzed by flow cytometry. The distributions of sperm in quadrants (Q_1–Q_4) of contour plot B were done using Consort 30 Revision D software. This analysis shows the proportion of 10,000 sperm cells that fall within each quadrant. The sperm cells that retained membrane integrity and functional mitochondria, as indicated by CDMFDA and R123 staining, were quantified as quadrant 2 (Q_2), whereas those cells that had lost membrane integrity and stained with PI were measured as quadrant 1 (Q_1). Actual percentages for each of the four quadrants for this cryopreserved sample are given on the right.

compensation to minimize this phenomenon. The incompleteness of this compensation is demonstrated by the fact that the CDMFDA–R123 population is quantified as quadrant 2 (Q_2; Fig. 2B,C,F) rather than quadrant 4 (Q_4; Fig. 2B). Quadrant 3, which contained cellular debris, exhibited a decreased amount of fluorescence for all three stains (Q_3; Fig. 2B). Quadrant 4 was quantified as those residual spermatozoa demonstrating only green fluorescent staining (CDMFDA and R123) without some crossover staining in the red channels (Q_4; Fig. 2B). The crossover into the red channels is such that only a few sperm remain in Q_4. This does not alter the validity of the combined stain because only live cells stain with CDMFDA or R123 and only dead cells exhibit strong PI staining without the green staining (Fig. 1A,E).

Statistical Analyses

The percentage values were arcsine transformed before statistical analyses were performed to control for skewness (TRANSPOSE procedure, SAS Institute, Cary, North Carolina). The analyses of the transformed means were carried out using ANOVA, TTEST, and the Waller–Duncan K-ratio t-test (SAS Institute). Simple correlations and linear regressions were carried out using the SAS CORR and REG procedures. The correlation data (Figs. 4–6) were plotted using PlotIT (Scientific Programming Enterprises, Haslett, Michigan).

Results

Sperm Motion Parameters

The mean values and standard deviations for the percentages of motile spermatozoa and sperm curvilinear velocities are given in Table 1. The overall means of 15 donors of sperm motility for the initial, 24-hour extended and thawed cryopreserved motility were 76.4 ± 15.1%, 39.9 ± 12.9%, and 44.2 ± 12.4%, respectively. The mean value for initial sperm motility for the 15 donors differed from the means of both the 24-hour stored ($P = 0.001$) and cryopreserved samples ($P = 0.001$). Differences ($P = 0.001$) among the 15 individual donors were noted for all three motility assessments; the initial determination (Mot-1), the 24-hour stored samples (Mot-2), and the cryopreserved samples (Mot-3).

The mean values for the velocities of spermatozoa were 47.3 ± 8.6, 35.5 ± 7.0, and 37.5 ± 8.8 for the initial, 24-hour, and cryopreserved samples, respectively. As was found for the motility values, the mean value for initial curvilinear velocity differed from the means of both the 24-hour stored ($P = 0.001$) and cryopreserved samples ($P = 0.001$). Differences among the donors were noted for all three velocity assessments ($P < 0.004$).

Flow Cytometry

Quadrant analyses of the flow cytometric data are provided in Table 2. The mean percentage and standard deviations of spermatozoa within each of the quadrants for the 24-hour stored samples ($Q_{1\text{–}4(24)}$) were 25.3 ± 7.1%, 55.6 ± 13.6%, 14.1 ± 8.4%, and 5.9 ± 3.2% for quadrants 1, 2, 3, and 4, respectively; whereas those for the cryopreserved samples ($Q_{1\text{–}4(CP)}$) were 51.8 ± 10.3%, 28.6 ± 10.2%, 18.1 ± 5.6%, and 1.5 ± 1.5% for quadrants 1, 2, 3, and 4, respectively. The means of the three replicates for the

Table 1. *Relative percentages and velocities of motile sperm as determined by computer-assisted semen analyses* for 15 human semen samples*

	Sperm motility†			Sperm velocity‡		
Donor	Mot-1	Mot-2	Mot-3	Vel-1	Vel-2	Vel-3
1	86.2	47.2	40.6	48.2	39.8	42.5
2	51.0	23.4	23.6	46.0	37.1	45.5
3	88.5	37.7	45.4	72.9	40.2	59.6
4	83.1	56.9	44.4	42.1	42.9	37.1
5	85.7	55.7	45.5	55.9	43.6	34.0
6	86.7	51.3	59.3	47.6	36.1	35.8
7	60.5	31.3	39.0	42.4	29.0	38.6
8	78.9	41.4	61.5	39.9	43.1	43.8
9	82.6	34.7	66.2	44.3	43.8	36.8
10	93.5	36.8	58.2	47.4	32.4	40.8
11	38.9	18.8	33.3	51.0	32.8	38.5
12	79.3	34.6	43.2	39.3	30.8	30.6
13	73.1	61.3	32.0	50.4	34.0	25.2
14	74.2	23.4	41.7	42.2	22.4	26.1
15	83.2	44.6	29.6	39.5	24.0	27.0
Mean	76.4 ± 15.1	39.9 ± 12.9	44.2 ± 12.4	47.3 ± 8.6	35.5 ± 7.0	37.5 ± 8.8

* CellSoft Clinical Module (CRYO Resources, New York, New York).
† Percentages of motile spermatozoa 20 minutes after ejaculation (Mot-1), after 24 hours storage (Mot-2), and in thawed, cryopreserved samples (Mot-3) ($n = 2$).
‡ Sperm velocities 20 minutes after ejaculation (Vel-1), after 24 hours storage (Vel-2), and in thawed, cryopreserved samples (Vel-3) ($n = 2$).

15 individual donors differed ($P < 0.05$) for all four quadrants, quadrants 1–4, for both the stored and cryopreserved samples. The overall means for quadrant 1 differed ($P < 0.05$) when the data from the stored samples ($Q_{1(24)}$) were compared with that obtained for the cryopreserved samples ($Q_{1(CP)}$). Thus, cryopreservation increased the proportion of spermatozoa staining with PI.

The mean percentages of spermatozoa with functional membranes and mitochondria, as identified by CDMFDA and R123 staining (quadrant 2), were 55.6 ± 13.6% for the 24-hour extended samples ($Q_{2(24)}$) and 28.6 ± 10.2% for the thawed cryopreserved samples ($Q_{2(CP)}$; Table 2). Not only did the group means for ($Q_{2(24)}$) and ($Q_{2(CP)}$) differ ($P = 0.001$), but the means of individual donors demonstrate that cryopreservation resulted in an easily identifiable change in the relative fluorescence distribution of the samples (Table 2; Fig. 3). The effects of cryopreservation on the relative distribution and size of the fluorescently stained sperm populations is evident among the four of the semen donors shown in Figure 3. Such a

Table 2. *The proportions of spermatozoa that were quantified by flow cytometric quadrant analyses* for 15 human semen samples*

Donor	$Q_{1(24)}$	$Q_{2(24)}$	$Q_{3(24)}$	$Q_{4(24)}$	$Q_{1(CP)}$	$Q_{2(CP)}$	$Q_{3(CP)}$	$Q_{4(CP)}$
1	22.9	58.7	8.9	9.5	47.5	36.0	13.2	3.4
2	12.9	71.4	7.6	8.2	45.0	36.4	13.8	4.9
3	14.0	71.0	9.5	5.4	39.7	37.7	18.2	4.4
4	29.9	40.6	20.7	6.7	61.1	16.6	21.5	0.8
5	33.4	46.9	8.7	11.0	67.9	21.8	9.9	0.3
6	18.1	71.0	3.1	7.8	64.5	20.1	14.6	0.8
7	32.3	51.6	10.9	5.2	62.6	23.6	13.3	0.4
8	33.7	38.0	22.3	6.0	49.9	16.5	32.4	1.2
9	24.0	53.5	16.0	6.5	57.7	18.6	23.2	0.5
10	15.9	60.9	15.0	8.2	45.2	39.5	13.3	2.0
11	32.0	27.0	36.3	4.7	60.1	19.1	20.0	0.8
12	22.8	68.6	7.1	1.5	30.2	50.4	18.0	1.4
13	34.5	55.4	9.4	0.7	48.4	30.1	20.4	1.1
14	15.4	69.4	13.4	1.8	48.5	28.6	22.7	0.2
15	25.3	49.8	22.3	2.6	48.0	34.1	17.8	0.1
Mean	25.3 ± 7.1	55.6 ± 13.6	14.1 ± 8.4	5.9 ± 3.2	51.8 ± 10.3	28.6 ± 10.2	18.1 ± 5.6	1.5 ± 1.5

* Proportions of spermatozoa stained with carboxydimethylfluorescein diacetate and rhodamine 123 quantified by flow cytometry for 24-hour stored samples as $Q_{1(24)}$, $Q_{2(24)}$, $Q_{3(24)}$, and $Q_{4(24)}$ and for cryopreserved samples as $Q_{1(CP)}$, $Q_{2(CP)}$, $Q_{3(CP)}$, and $Q_{4(CP)}$ ($n = 3$). See Figure 2 for the quadrant analyses as carried out using Consort 30 software (C30 version D, Becton Dickinson, Sunnyvale, California).

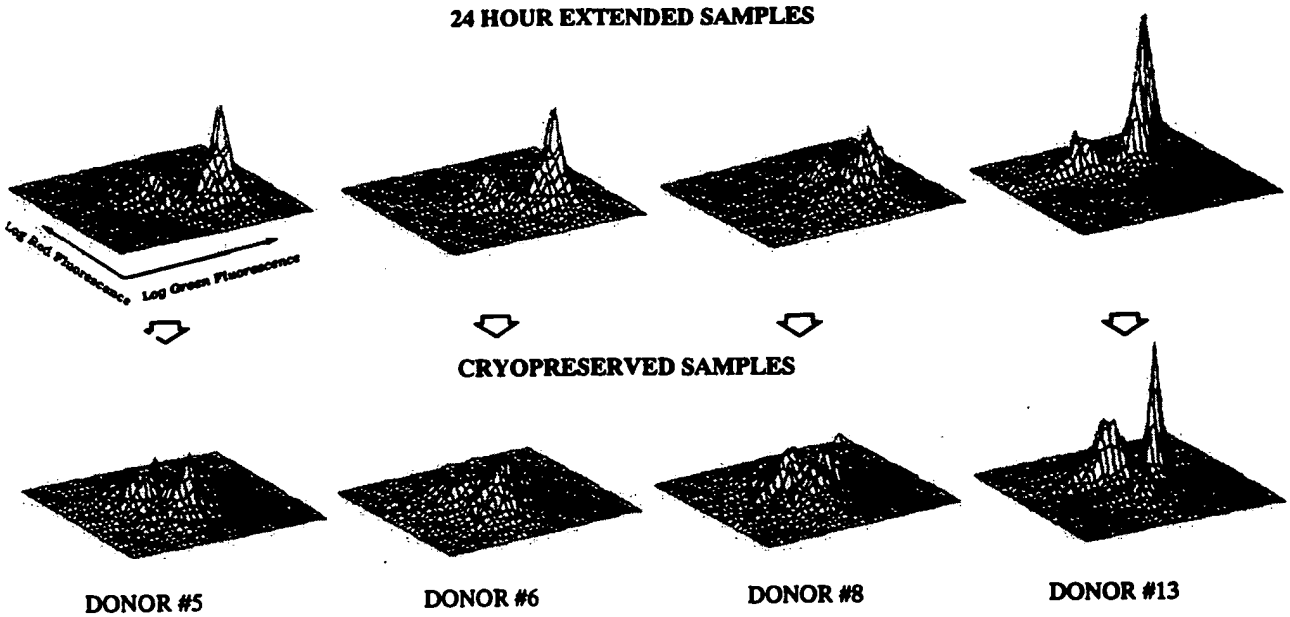

FIG. 3. Net plots showing changes in the relative distribution of sperm populations of four donors as the result of cryopreservation. Note that relatively little change occurred in the sample from donor #13, whereas the changes in the samples from donors #5, #6, and especially #8 were much greater. Cryopreservation appeared to cause a significant loss of membrane integrity and thus a corresponding redistribution of sperm within the flow cytometric fluorescence patterns.

comparative approach may be useful in quantifying individual differences among donors in their ability to undergo the cryopreservation process.

Variability was evident for quadrant 3 with the overall mean percentages of cells within this quadrant being 14.1 ± 8.4% and 18.1 ± 5.6% of the 24-hour stored and cryopreserved samples, respectively. These values were not different ($P > 0.05$).

Quadrant 4 contained the smallest proportion of spermatozoa, 5.9 ± 3.2% and 1.5 ± 1.5% for the 24-hour stored and cryopreserved samples, respectively. The difference between the stored and cryopreserved samples ($Q_{4(24)}$) vs. $Q_{4(CP)}$) was significant ($P < 0.05$). Considerable variability, however, occurred among the donors for this population.

Interrelationships among Sperm Motion and Flow Cytometry Parameters

Initial sperm motility (Mot-1) was positively correlated with both the motility of the 24-hour extended samples (Mot-2) ($r = 0.61$, $P = 0.02$) and that for the thawed cryopreserved sperm aliquots (Mot-3) ($r = 0.58$, $P = 0.023$) (Table 3). Initial sperm velocity (Vel-1) was positively correlated with the velocity of the thawed, cryopreserved samples (Vel-3) ($r = 0.61$, $P = 0.015$) but not with the velocity obtained after the 24-hour storage period (Vel-2) ($r = 0.34$, $P = 0.21$). The velocity of the 24-hour stored spermatozoa (Vel-2), however, was positively correlated with the thawed cryopreserved spermatozoa (Vel-3) ($r = 0.52$, $P = 0.045$; Table 3). In addition, the velocity of the spermatozoa in the 24-hour extended samples was correlated with the proportion of sperm cells in $Q_{4(24)}$ (spermatozoa retaining green fluorescence with little red fluorescence; $r = 0.69$, $P = 0.004$; Table 3), whereas the velocity of the cryopreserved samples (Vel-3) was correlated with the proportion of sperm that had retained green fluorescence in the same thawed, cryopreserved samples ($Q_{4(CP)}$) ($r = 0.74$, $P = 0.002$; Table 3). Quadrant 4 was the only flow cytometric parameter that was correlated with any of the sperm motion values.

Initial sperm motility (Mot-1) was, at least partially, useful in estimating what the motility would be for the same samples after extension in TEST–yolk and storage for 24 hours at 5°C (Mot-2) (Fig. 4A, $r = 0.61$, $P = 0.015$) and after cryopreservation (Mot-3) (Fig. 4B, $r = 0.58$, $P = 0.023$). Initial sperm velocity (Vel-1) was correlated with the velocity of the spermatozoa in the thawed, cryopreserved samples (Vel-3) (Fig. 5A, $r = 0.61$, $P = 0.02$) and also to the velocity of the gametes in the 24-hour extended samples (Vel-2) but to a lesser degree (Fig. 5B, $r = 0.52$, $P < 0.05$).

The number of dead spermatozoa in cryopreserved samples (i.e., cells that stained only with PI; $Q_{1(CP)}$) was negatively correlated with the number of spermatozoa in cryopreserved samples with intact plasma membranes and functional mitochondria (i.e., cells that retained the maximum amount of CDMFDA and R123; $Q_{2(CP)}$; $r = -0.81$; $P = 0.001$) but not to that proportion of spermatozoa that

Table 3. *Simple correlation coefficients among the human sperm populations identified by computer-assisted sperm motility analyses (CASA) and quadrant analyses (Q_{1-4}) of flow cytometric data on fluorescently stained human spermatozoa**

Sperm motility†/ velocity‡/ populations§	Sperm populations§/velocity‡/motility†												
	$Q_{4(CP)}$	$Q_{3(CP)}$	$Q_{2(CP)}$	$Q_{1(CP)}$	$Q_{4(24)}$	$Q_{3(24)}$	$Q_{2(24)}$	$Q_{1(24)}$	Vel-3	Vel-2	Vel-1	Mot-3	Mot-2
Mot-1	−0.05	−0.05	0.18	−0.09	0.23	−0.42	0.32	−0.20	0.02	0.24	0.12	0.58‖	0.61‖
Mot-2	−0.19	−0.09	−0.17	0.23	0.22	−0.31	−0.09	0.39	−0.21	0.40	0.09	0.16	
Mot-3	−0.25	0.31	−0.33	0.20	0.31	−0.07	−0.00	−0.04	0.18	0.44	−0.03		
Vel-1	0.50	−0.20	0.11	0.00	0.22	−0.18	0.19	−0.22	0.61‖	0.34			
Vel-2	0.28	0.11	0.39	0.25	0.69‖	−0.08	−0.23	0.21	0.52‖				
Vel-3	0.74‖	−0.05	0.06	−0.12	0.49	−0.05	0.11	−0.34					
$Q_{1(24)}$	−0.58‖	0.25	0.54‖	0.34	−0.07	0.40	−0.81*						
$Q_{2(24)}$	0.48	−0.35	0.63‖	−0.35	−0.11	−0.82*							
$Q_{3(24)}$	−0.34	0.49	−0.41	0.07	−0.14								
$Q_{4(24)}$	0.28	−0.40	−0.30	0.47									
$Q_{1(CP)}$	−0.48	−0.21	−0.81‖										
$Q_{2(CP)}$	0.51	−0.37											
$Q_{3(CP)}$	−0.24												

* Semen samples from 15 men were analyzed by flow cytometry in triplicate and for CASA in duplicate. Department of OB-GYN, Michael Reese Hospital, Chicago, Illinois (n = 15).

† The percentage of motile sperm using CRYO Resources CellSoft CASA. Motility was examined within 1 hour after ejaculation (Mot-1), after 24 hours storage at 5°C (Mot-2), and after thawing identical cryopreserved samples (Mot-3) (n = 2).

‡ The velocities of motile sperm as determined by CASA using CRYO Resources CellSoft CASA. Velocities were examined within 1 hour after ejaculation (Vel-1), after 24 hours storage at 5°C (Vel-2), and after thawing identical cryopreserved samples (Vel-3) (n = 2).

§ Flow cytometric quadrant analyses were done after 24 hours storage ($Q_{1(24)}$, $Q_{2(24)}$, $Q_{3(24)}$, and $Q_{4(24)}$) and on cryopreserved samples ($Q_{1(CP)}$, $Q_{2(CP)}$, $Q_{3(CP)}$, and $Q_{4(CP)}$).

‖ $P < 0.05$.

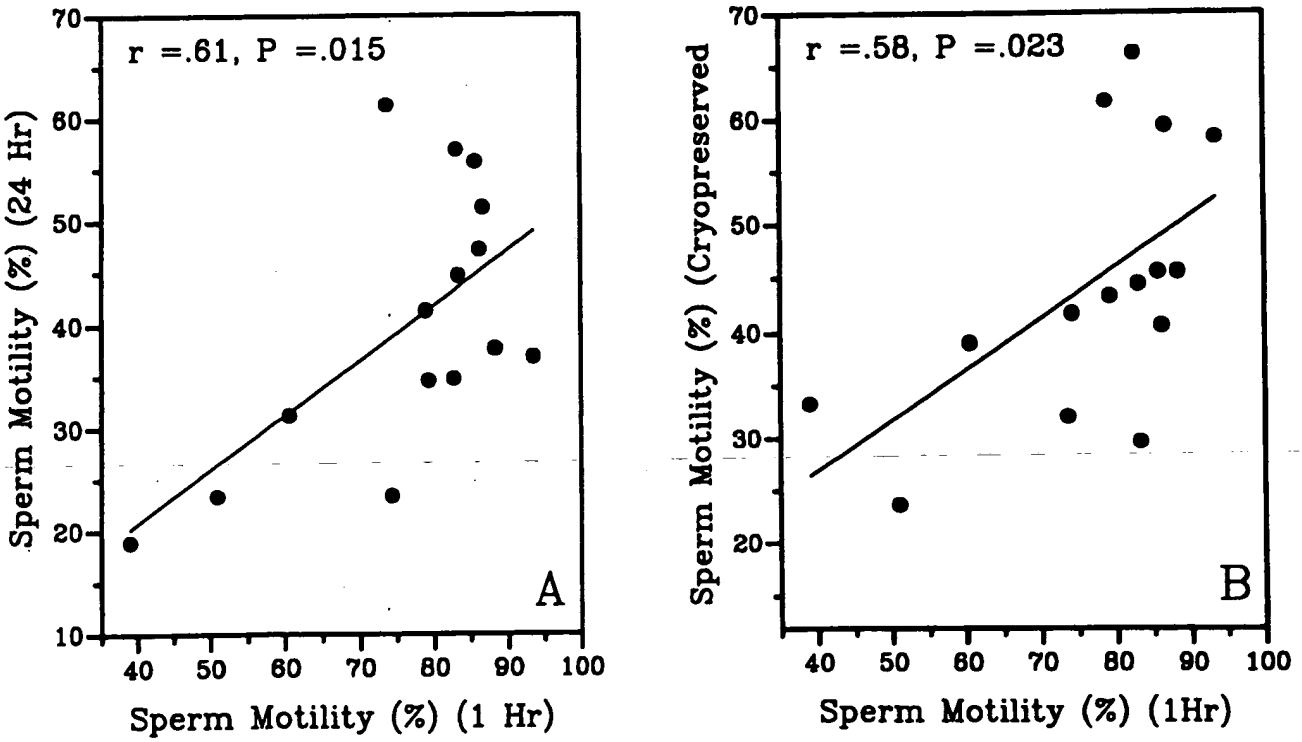

FIG. 4. Linear regression plots and correlations between the percentage of motile spermatozoa found initially and that observed after storage in TEST–yolk buffer at 5°C (**A**) (r = 0.61, P = 0.015). Initial sperm motility was also correlated with the post-thaw motility of the cryopreserved samples (**B**) (r = 0.58, P = 0.023) (n = 15).

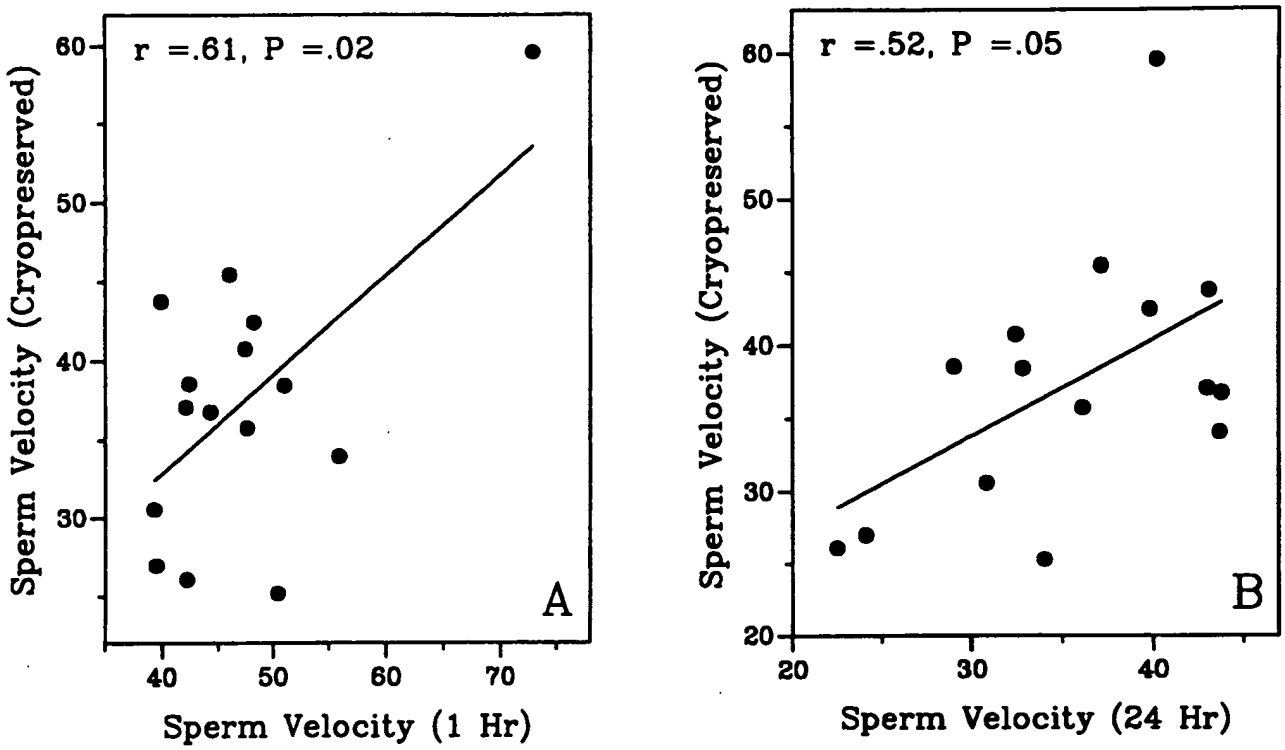

FIG. 5. Linear regression plots and correlations showing the positive relationship between the post-thaw velocity of cryopreserved sperm samples with the initial spermatozoal velocity (A) (r = 0.61, P = 0.02) and with the velocity observed for aliquots of same samples that were not frozen but stored in TEST–yolk buffer for 24 hours (B) (r = 0.52, P = 0.05) (n = 15).

resisted PI $Q_{4(CP)}$ ($r = -0.48$, $P < 0.05$; Table 3). The proportion of spermatozoa in quadrant 2 for the thawed, cryopreserved samples ($Q_{2(CP)}$) was not correlated with the proportion of sperm cells in quadrant 4 ($Q_{4(CP)}$) ($r = 0.53$, $P < 0.05$; Table 3). In cryopreserved samples, the proportion of dead spermatozoa, i.e., those staining with PI ($Q_{1(CP)}$), was negatively correlated with the proportion of spermatozoa with functional membranes and mitochondria (CDMFDA and R123 stained) ($Q_{2(CP)}$; $r = -0.81$, $P = 0.001$; Table 3). This relationship was also evident in the 24-hour extended samples ($Q_{1(24)}$ vs. $Q_{2(24)}$, $r = -0.81$, $P = 0.001$; Table 3).

Flow cytometry demonstrated some interesting interrelationships among the sperm populations. As expected, the proportion of spermatozoa that stained with PI ($Q_{1(24)}$) was negatively correlated with those cells stained with CDMFDA and R123 ($Q_{2(24)}$) (Fig. 6A, $r = -0.81$, $P = 0.001$). In addition, the proportion of spermatozoa in the 24-hour extended samples stained with CDMFDA and R123 ($Q_{2(24)}$), and the same populations in thawed, cryopreserved spermatozoa ($Q_{2(CP)}$) were correlated (Fig. 6B, $r = 0.63$, $P = 0.011$). The proportion of spermatozoa in the 24-hour extended samples ($Q_{4(24)}$) (i.e., spermatozoa that retained green fluorescence with low red fluorescence) and the same population in cryopreserved samples $Q_{4(CP)}$ were not correlated ($r = 0.28$, $P < 0.05$).

Discussion

A reduction in both sperm motility and curvilinear sperm velocity was noted when spermatozoa were extended and stored at 5°C for 24 hours as previously demonstrated by several others (Mack and Zaneveld, 1987; Bielfeld et al, 1990). The flow cytometric analyses verified that cryopreservation resulted in a reduced number of spermatozoa with intact membranes. Even though the initial proportions of spermatozoa with intact membranes for the ejaculated samples were not determined, the proportions of sperm cells in 24-hour extended samples possessing intact membranes were approximately 50% greater than those found for the thawed, cryopreserved samples from the same men (Table 2; $Q_{2(24)}$ and $Q_{2(CP)}$). This reduction in membrane integrity of thawed–cryopreserved spermatozoa as compared to the 24-hour extended samples confirms the data of Mack and Zaneveld (1987), Critser et al (1985), and McLaughlin et al (1992). Initial sperm motility was approximately 30–50% greater than the motilities found for either the 24-hour extended or thawed, cryopreserved samples (Table 1; Mot-1, Mot-2, and Mot-3).

Our preliminary data (Garner et al, 1991) had suggested that the parameters measured by the flow cytometer were not directly related to the sperm motion parameters mea-

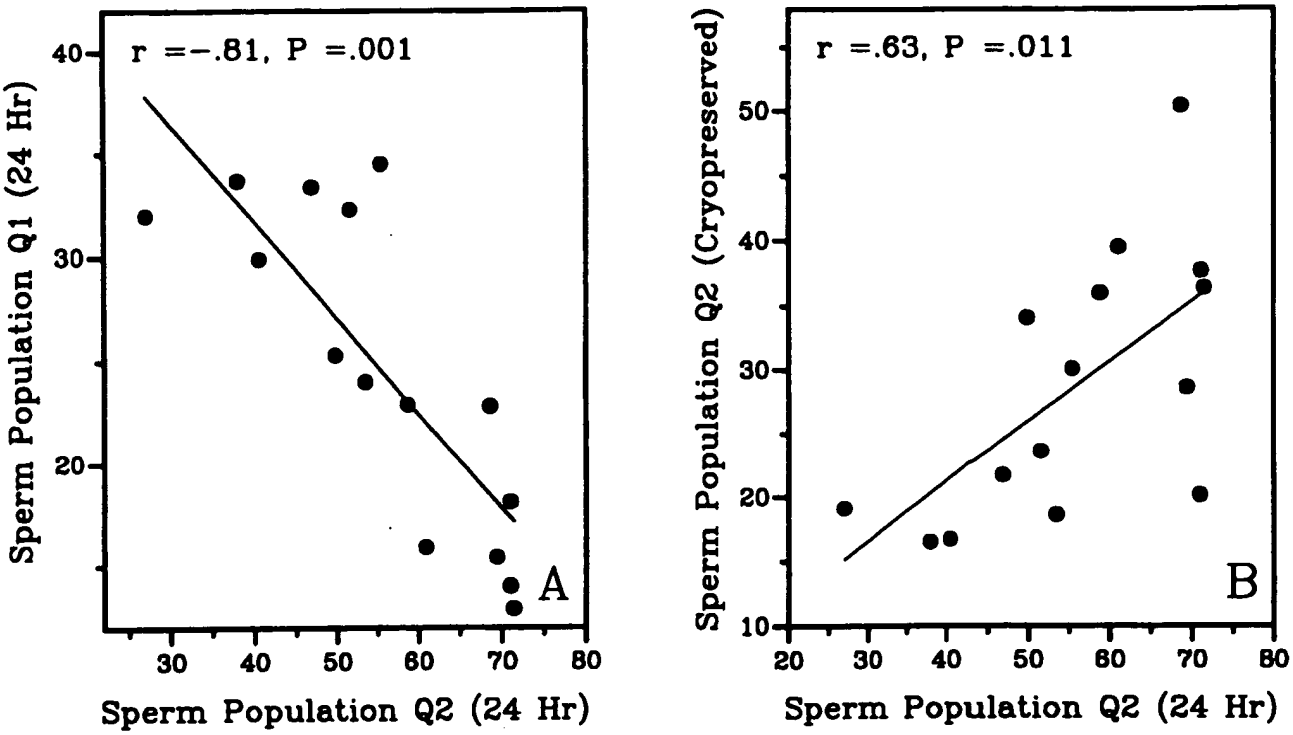

FIG. 6. Linear regression plot and correlation between the number of dead spermatozoa (i.e., high red fluorescence from PI staining) in the 24-hour extended samples and the proportion of those cells in 24-hour extended samples retaining the CDMFDA and R123 (A) ($r = 0.81$, $P = 0.001$). The linear regression positive plot and correlation values in B show the relationship between the proportion of spermatozoa in 24-hour extended samples and those in cryopreserved samples that retained CDMFDA and R123 ($r = 0.63$, $P = 0.011$) ($n = 15$).

sured by CASA. However, subsequent data confirmed that at least one relationship existed among the sperm quality parameters measured by these two instruments. Sperm velocity after 24 hours storage (Vel-2) was correlated with the percentage of spermatozoa that had retained the green fluorescence after 24 hours storage in TEST-yolk medium $Q_{4(24)}$. In addition, the velocity of the cryopreserved samples was correlated with the sperm population that retained green fluorescence while excluding the PI ($Q_{4(CP)}$). This relationship suggests that the retention of membrane integrity is required for a sperm cell to maintain adequate swimming velocity or that membrane integrity and mitochondrial function decrease at a rate similar to that of sperm velocity. McLaughlin et al (1992) concluded that membrane rupture was not the sole cause of the decrease in sperm motility, but that at least part of the decrease in the proportion of motile spermatozoa and the velocity of each was caused by something other than cryopreservation. The correlation between the proportion of thawed, cryopreserved spermatozoa retaining green fluorescence and sperm velocity in the present study, however, suggests that some interaction must exist between sperm velocity parameters and the sperm population identified by CDMFDA and R123 staining ($Q_{4(CP)}$; Table 3).

The CASA data indicated that both sperm motility and velocity decreased during storage in TEST-yolk medium with diluted seminal plasma and that this decrease was only weakly related to mitochondrial function and/or sperm plasma membrane integrity. In contrast, cryopreserved spermatozoa retained their motility (Mot-3) and velocity (Vel-3) similar to that shown for the 24-hour extended samples (Table 1, Mot-2 and Mot-3; Vel-2 and Vel-3). The cryopreserved samples relative to the 24-hour extended samples exhibited a marked loss of plasma membrane integrity and/or mitochondrial function. This was indicated by the decreased proportion of sperm cells in quadrant 2 $Q_{2(CP)}$. A relatively high proportion of the spermatozoa in the thawed, cryopreserved samples did retain the CDMFDA and R123 (Table 2; Fig. 6). Samples from two of the donors, numbers 4 and 8, were considered to be of poor quality because only about 16% of the spermatozoa had retained high green fluorescence and, thus, membrane integrity (Table 2; Fig. 3, donor #8).

The sperm velocities of both the initial (Vel-1) and the 24-hour stored (Vel-2) samples were predictive of the velocity of the cryopreserved samples (Vel-3). This relationship, along with that of Ginsburg et al (1990), suggests that sperm velocity is a better indicator of potential fertility than other sperm motion parameters. The cryopreservation process appeared to have had a greater impact on the reduction of sperm motility than it did for sperm velocity (Table 1, Mot-1 and Vel-3). These data are in

agreement with that of Keel and Webster (1989). Marshburn et al (1992) reported that the analyses of sperm motion characteristics, especially curvilinear velocity, after thawing and washing the cryopreserved spermatozoa were a better indicator of fertility outcome after intrauterine insemination than CASA analysis of the fresh semen. The importance of sperm velocity measurements is demonstrated by the interrelationships between the velocity and green fluorescence of the 24-hour stored samples (Vel-2 and $Q_{4(24)}$, $r = 0.69$) and the same measurements for the cryopreserved samples (Vel-3 and $Q_{4(CP)}$, $r = 0.74$). Furthermore, cryopreservation appeared to affect plasma membrane integrity and mitochondrial function as indicated by the decrease in the proportion of thawed, cryopreserved spermatozoa retaining the CDMFDA and R123 stains ($Q_{2(CP)}$ and $Q_{4(CP)}$) (Table 2; Fig. 6). The staining patterns observed in the present study for human spermatozoa are also seen in thawed, cryopreserved bovine spermatozoa when stained with CDMFDA, PI, and R123 and analyzed using flow cytometry (Garner et al, 1992; Ericsson et al, 1993).

The proportion of spermatozoa retaining CDMFDA and R123 in the 24-hour stored sperm samples could be used, at least partially, to estimate what proportion of spermatozoa are likely to survive cryopreservation. This concept is supported by the relationship ($r = 0.61$; Table 3) between the sperm population that retained membrane integrity and mitochondrial function in 24-hour extended sperm samples ($Q_{2(24)}$) and the same population in the cryopreserved samples ($Q_{2(CP)}$). A comparison of the within-quadrant analyses showed that the proportion of dead spermatozoa (Q_1, PI positive) in both the stored ($Q_{1(24)}$ vs. $Q_{2(24)}$, $r = -0.81$, $P = 0.001$) and cryopreserved samples ($Q_{1(24)}$ vs. $Q_{2(CP)}$, $r = -0.54$) were inversely related to the number of spermatozoa that had retained CDMFDA and R123. As would be expected, a comparison of quadrants in cryopreserved samples showed that the proportion of dead spermatozoa ($Q_{1(CP)}$, PI positive) in cryopreserved samples was negatively correlated ($r = -0.81$) to the proportion that had survived cryopreservation ($Q_{2(CP)}$).

Bielfeld et al (1990) showed that centrifugation increased the proportion of spermatozoa undergoing acrosome reactions. The samples were not centrifuged in the present study to eliminate this problem and to ensure against processing and medium inconsistencies among treatments (Mack and Zaneveld, 1987). This simplified processing approach, however, did not eliminate possible experimental effects due to the presence of seminal plasma. It should be noted that both sperm motility and velocity were, on average, less for 24-hour stored samples than for the cryopreserved samples (Table 1). The presence of seminal plasma also could account for the similarities noted among the 24-hour extended and cryopreserved sperm samples for sperm motility and velocity (Table 1). No comparisons, however, were made between samples with or without seminal plasma in the present study.

The regression analyses showed that the degree of linearity was marginal for most of the variables investigated in this study. The variation among these 15 donors was large (Tables 1, 2; Fig. 3). A larger sample size would tend to increase the linearity by minimizing the variation among the donors that was created by these extremes. The only sperm motion parameter that was correlated with one of the flow cytometrically identified populations (Q_4) was curvilinear velocity in samples stored 24 hours ($r = 0.69$) and after thawing cryopreserved samples ($r = 0.74$). Flow cytometry and CASA measurements appear to be, for the most part, independent. Although we amply demonstrated the utility of using flow cytometry to assess several sperm function parameters in both 24-hour stored and in cryopreserved samples of human donor semen, additional research is warranted to assist clinical andrology laboratories in predicting the effect of cryopreservation on sperm motion parameters, plasma membrane integrity, and mitochondrial function from the initial examination of ejaculated samples. In addition, the data resulting from these studies show that further research will be needed to determine if any other combinations of seminal quality parameters are related to the potential fertility of that sample.

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Offspring from inseminations with mammalian sperm stained with Hoechst 33342, either with or without flow cytometry

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Summary

The technique of vital staining with a fluorochrome and subsequent flow cytometry has been employed to investigate the DNA content of living sperm. There have been several reports of chromosomal damage caused by staining cells with a bis-benzimidazole dye, Hoechst 33342, either with or without subsequent flow cytometry. Therefore the use of this technique might be expected to affect adversely the fertilising capacity of inseminated sperm, or to result in the production of congenital deformities in the young. Insemination experiments to date have resulted in the production of more than 400 offspring in four species, including 5 successive generations of rabbits, where stained or sorted sperm were used. All progeny were observed to be normal by anatomical criteria. There was no evidence to suggest that sperm treated in this manner were unable to fertilise oocytes in vivo or that embryonic development was affected.

The technique of flow cytometric analysis and separation of sperm populations stained with a fluorochrome has been reported by several groups (Pinkel et al., 1982; Garner et al., 1983; Keeler et al., 1983; Johnson, 1986; Johnson et al., 1987a,b; Morrell et al., 1988). Pinkel et al. (1982) stained fixed sperm with either ethidium bromide and mithramycin or DAPI (4′,6′-diamidino-2-phenylindole), while Garner et al. (1983, 1984) used DAPI in their experiments. Keeler et al. (1983) used the bis-benzimidazole dye Hoechst 33342 as a semi-quantitative vital fluorescent DNA (deoxyribonucleic acid) stain and recovered motile sperm after flow cytometry. Several publications refer to the use of Hoechst 33342 for fixed sperm (Johnson, 1984, 1986; Johnson and Pinkel, 1986; Johnson et al., 1987a) or for sperm which had been sonicated (Johnson et al., 1987b). The production, over a period of 5 years, of viable young after inseminations employing sperm stained vitally with Hoechst 33342 and subjected to flow cytometry, was reported by Morrell et al. (1988).

While it has been argued on physico-chemical grounds that bis-benzimidazole dyes have little effect on DNA structure (Muller and Gautier, 1975; Dickerson et al., 1985), Libbus et al. (1987) and Smith et al. (1988) have observed chromosomal abnormalities after vital staining with

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Hoechst 33342. Libbus and colleagues (1987) stained sperm of the vole *Microtus oregoni* for flow sorting and used the technique of microinjection in hamster oocytes to assess chromosomal damage in both stained and sorted sperm. They concluded that while the treatments and manipulations to which they subjected the vole sperm nuclei were responsible for chromosomal aberrations, including chromatid breaks and exchanges, a substantial contribution to chromosome damage was made by the exposure of the stained cells to laser irradiation (long UV) during flow cytometry. However no assessment was possible of the effects of staining and sorting of sperm on live offspring. In contrast to these results Martin et al. (1988) found that not only does sonication cause chromosomal abnormalities in unstained sperm but that microinjection itself increases the frequency of such aberrations.

Smith et al. (1988) showed that the occurrence of DNA strand breakages in a particular mouse cell line was correlated with the concentration of Hoechst 33342 used to stain the cells. Conover and Gwatkin (1988), who summarised the use of Hoechst 33342 in various aspects of in vitro fertilisation work, reported the use of this fluorochrome to detect fusion between mammalian gametes. If the use of Hoechst 33342 for the staining of gametes or zygotes is likely to increase in the future, it is important to ascertain whether this fluorochrome does cause chromosomal damage of importance in the live animal.

This report describes the results to date of inseminations with sperm stained with Hoechst 33342 in 4 species. These sperm were either stained but not passed through the flow cytometer ('stained') or were stained and subjected to flow cytometry ('sorted'). The objectives in sorting sperm were to extend the work reported in our preliminary study on pre-conceptual sex selection by separating X- and Y-chromosome-bearing sperm according to DNA content (Morrell et al., 1988). To maximize the chance of detecting possible adverse effects of the use of Hoechst 33342 we set out to breed successive generations of rabbits derived from treated sperm. The choice of rabbits for this breeding study was based on their convenience for laboratory study, the relatively short gestation and generation interval compared with farm animal species, and the production of litters of offspring.

Materials and methods

Semen collection and sperm preparation

Bull, ram and buck semen were collected by means of an artificial vagina appropriate to the species. Bull semen was diluted with an equal volume of UHT milk medium (Morrell et al., 1988) at 30 °C; the semen was diluted further by adding 200 µl of diluted semen to 10 ml UHT milk medium containing 1 µg/ml Hoechst 33342. Ram ejaculates were diluted in the same manner except that egg citrate medium (Willett and Salisbury, 1942) was used and the concentration of Hoechst 33342 was increased to 2 µg/ml. Each ejaculate of buck semen was diluted to 5 ml with egg citrate medium or modified egg citrate medium containing either 10% or 5% v/v egg yolk, at 30 °C. The diluted semen was stained with 4 µg/ml Hoechst 33342. After staining for at least 2 h in the dark at room temperature (20–22 °C) sperm were used for insemination experiments or were sorted by flow cytometry (Morrell et al., 1988).

Boar semen, diluted in Kiev medium (Plishko, 1965), was sent from a boar stud by express parcel delivery on the day of collection. The diluted semen was stained with 2 µg/ml Hoechst 33342 overnight at approximately 16–18 °C before sorting.

Synchronisation and insemination procedures

The oestrous cycles of heifers were synchronised by the administration of 500 µg cloprostenol (Estrumate, ICI) on 2 occasions 11 days apart. They were inseminated 55 and 79 h after the second injection using 1–5 million sperm. In the majority of cases pregnant heifers were aborted after 4–5 months gestation but in 4, pregnancy was allowed to proceed to establish the normality of the full-term calf.

Ewes were prepared for insemination by the insertion of progestagen sponges (Chronogest; Intervet) for 12 days followed by the intramuscular administration of 400 IU serum gonadotrophin (Folligon; Intervet) at the time of sponge removal. Intrauterine insemination was performed by the

laparoscopic technique of Killen and Caffery (1982). 5 million sperm were deposited directly in each uterine horn. Pregnancy was indicated by failure to return to oestrus 16–17 days post insemination and was confirmed by ultrasound examination at 60 days post insemination (Ginther and Pierson, 1983).

Female rabbits which were thought to be receptive to the male, on the basis of the colour of the vulva and behavioural changes, were inseminated with either 1–9 million sorted sperm, or up to 250 million stained sperm. Sperm for insemination were resuspended in 0.5 ml sodium citrate solution (2.9% w/v, adjusted to pH 6.7 with 10% citric acid). Several hours later the females were mated to a vasectomised buck to induce ovulation. The offspring from these inseminations were kept to maturity and were used for similar insemination experiments.

The synchronisation of gilts, supplied by a commercial pig breeding company, was achieved by the oral administration of 20 mg altrenogest (Regumate porcine; Hoechst Animal Health) for 18 consecutive days. 7 million sperm were placed directly into the oviducts of an anaesthetised animal using a surgical technique (Polge et al., 1970). Pregnancy was indicated by failure to return to oestrus.

Assessment of offspring

The normality of the offspring, using gross anatomical criteria, was determined at birth or at the time of induced abortion in the heifers. Further assessments of the offspring were made at regular intervals as they matured. In addition post mortem examinations were performed on any rabbits which died.

Experiments and results

The results of the insemination experiments employing either stained or sorted sperm are shown in Table 1. Live young were produced in all 4 species and in the resulting 421 offspring there was no evidence of any gross anatomical abnormalities. However conception rates did appear to be low, possibly due to an effect of sperm treatment.

TABLE 1

INSEMINATIONS WITH STAINED OR SORTED SPERM

Species	Sperm treatment	Number of animals		
		Inseminated	Pregnant	Born [a]
Cattle	stained	4	1	1
Cattle	sorted	199	43	40
Rabbits	stained	118	41	128
Rabbits	sorted	357	73	240
Pigs	sorted	2	2	9
Sheep	sorted	22	2	3
Total				421

Note: the ratio of the number pregnant to the number inseminated gives the conception rate of these females.
'stained', sperm treated with Hoechst 33342; 'sorted', stained sperm subjected to flow cytometry.
[a] Includes aborted bovine foetuses.

To investigate which sperm treatment might be responsible for the low conception rate, a separate series of insemination experiments was performed in rabbits. Procedures which might have contributed towards loss of viability of the sperm were identified as (1) the delay between collecting the ejaculate and inseminating females (some 5–12 h); (2) the small numbers of sperm available for insemination; (3) the use of Hoechst 33342 to stain the sperm; and (4) the flow cytometric procedure itself. 4 groups of rabbits were inseminated with aliquots of 10 million sperm treated as follows:

Group 1: sperm from an ejaculate diluted immediately after collection and inseminated immediately;

group 2: sperm from semen kept in the dark at room temperature (20 °C) for 5 h before insemination;

group 3: sperm from a stained ejaculate stored as described for group 2;

group 4: sorted sperm inseminated after flow cytometry (5–6 h after collection).

Control inseminations (for groups 1, 2 and 3) were performed at the same time using the remaining half of the ejaculates prepared as described above. Based on an average rabbit ejaculate containing 500 million sperm (Napier, 1963), it is assumed that each control inseminate contained approximately 250 million sperm.

TABLE 2

INSEMINATIONS TO DETERMINE THE CAUSE OF DECREASED CONCEPTION RATES IN RABBITS

Group	Number of sperm (millions)	Conception rate	Litter size (average)
1	250	4/10	4
	10	3/20	2
2	250	3/6	4
	10	2/12	3
3	250	2/4	4
	10	2/12	3
4	10	8/49	1.6

Conception rate = number of animals pregnant/number inseminated. Group 1, diluted sperm inseminated immediately; group 2, diluted sperm held in the dark for 5 h before insemination; group 3, diluted sperm stained and stored as for group 2; group 4, sorted sperm inseminated 5–6 h after collection.

The results, shown in Table 2, indicated that the conception rates were not different when the same number of sperm were inseminated. This seems to be true regardless of the treatment to which the sperm had been subjected although there was a considerable reduction in conception rate and litter size between insemination doses of 250 million sperm and 10 million sperm.

Table 3 shows a comparison of the conception rates and average litter sizes for stained or sorted sperm over several successive generations. Since there was a marked decrease in conception rate where only small numbers of sperm were inseminated (Table 2), results of inseminations involving 250 million sperm are presented in addition to those using 1–9 million sperm (Table 3). It can be seen that the conception rate and litter size were lowest for the first generation of rabbits derived from stained/sorted sperm but that the situation improved for subsequent generations. In addition it was observed that there was a high incidence of perinatal death in those litters consisting of only one offspring, which correlated positively with increased gestation length. Table 4 shows the gestation lengths for females carrying litters of varying sizes. The length of gestation was considerably longer in does carrying singletons (35 days on 26 observations) compared with those bearing litters of 6 or more (32.7 days on 30 observations).

TABLE 3

COMPARISON OF CONCEPTION RATE AND AVERAGE LITTER SIZE FOR STAINED OR SORTED SPERM IN 2 CONCENTRATIONS OVER SEVERAL GENERATIONS

Generation	Sperm treatment [a]					
	Unstained		Stained		Sorted 1–9	Natural mating 500 [b]
	250	1–9	250	1–9		
0	2/11 (3)	3/20 (1.6)	6/42 (3.8)	1/3 (1)	7/98 (3.5)	5/6 (5)
1	2/4 (7)	2/8 (3)	3/6 (2)	4/26 (5)	11/43 (1/.7)	–
2	4/5 (4.25)	(0/4 –	8/16 (5.3)	1/9 (4)	10/50 (3.5)	4/5 (3)
3	3/5 (3)	0/3	1/2 (4)	0/2	12/38 (6./2)	2/2 (9)
4	15/27 (6)	–	–	–	21/52 (5)	1/1 (8)
5	1/1 (4)	–	–	–	–	–

[a] Approximate number of sperm (millions).
[b] An average buck ejaculate (Napier, 1963).
Conception rate = number pregnant/number inseminated.
(*n*), average litter size.
Generations 1–4 are the progeny of stained or sorted sperm.

TABLE 4

RELATIONSHIP BETWEEN LITTER SIZE AND AVERAGE GESTATION LENGTH

Size	Gestation length (days)		Number of litters
	Average	Range	
1	35	32–40	26
2	34	30–36	19
3	33.5	31–35	22
4	33.5	32–36	16
5	33.5	33–35	15
6	32.5	32–35	9
7	33	33–35	7
8	33	32–34	7
9	32.5	32–33	2
10	33		1
11	32		1
12	33		1
13	32		2

Note: day of insemination is counted as day 1.
Size, number of progeny in each litter.

TABLE 5

EFFECT OF HETEROSIS IN INSEMINATIONS INVOLVING CROSSES BETWEEN 2 ISOLATED STRAINS OF RABBITS

Cross	Conception rate	Litter size
New male × old female	10/41 (25%)	3
Old male × new female	8/17 (49%)	6
Old male × old female	6/36 (17%)	1.5
New male × new female	2/2 (100%)	6

'old', original line; 'new', introduced line.
Conception rate = number pregnant/number inseminated.

The fertility of the rabbits appeared to be poor in the first 2 generations in this breeding study. Since a degree of inbreeding was likely in our small colony, it was decided to introduce rabbits from a different source. The results of crosses between the 2 lines are shown in Table 5. Both conception rate and litter size were greatly improved in crosses involving females from the new colony compared with females from the original colony. It seems highly likely that the low fertility observed in our colony was due to a degree of inbreeding rather than the use of stained or sorted sperm.

Discussion

The low conception rate we experienced initially in all species inseminated with sorted sperm could have been due to several factors. These include the low sperm numbers available, sub-optimal timing of insemination in synchronised animals, ageing of sperm in the time between collection and insemination (some 5–12 h), and possible damage to sperm caused by staining and/or exposure to long UV during the sorting process.

The requirement to use the flow cytometer determined the time of insemination. Although heifers and ewes could be synchronised to be in oestrus on the right day, individual variation would affect the exact time of ovulation following the synchronisation treatment (Deas et al., 1979). Therefore it was inevitable that some inseminations would occur at a less favourable time relative to ovulation. Due to a shortage of experimental animals, non-pregnant heifers were subjected initially to repeated synchronisation treatments. However this appeared to have an adverse effect on conception rates (unpublished data); recently we have avoided re-use whenever this is practicable.

3 out of 43 heifers which were observed to be pregnant at days 28–35 of pregnancy, either by ultrasonic examination or by rectal palpation, were later found to have resorbed the foetus. This figure is similar to the drop of 15% between the 30- and 60-day non-return rate and the actual calving rate, estimated by the Milk Marketing Board (quoted by Laing, 1979).

Sperm numbers per inseminate were governed by the rate of sorting and consequently were much lower than would be considered for commercial artificial insemination. In heifers we inseminated 1–5 million sperm which is lower than the commercial dose rate of 20 million. Our ewes received a total of 5–10 million sperm in each uterine horn as opposed to approximately 90–100 million used by Killen and Caffery (1982). For rabbits Hafez (1970) quotes an optimal dose of 20–50 million sperm whereas we were able to use only 1–9 million sorted sperm for a similar method of insemination.

In view of the small sample size the apparently high conception rate of the pig inseminations is likely to be due to chance since in surgical inseminations using unstained sperm, the same operators achieved a pregnancy rate of only 2/22 (9%). However the result shows that viable young can be obtained from sorted sperm in this species.

The results from the second series of rabbit inseminations (Table 2), although involving small numbers of animals, did not indicate any difference in conception rates between treatment groups. However, while there was a considerable difference in conception rates where 10 million sperm compared to 250 million sperm were used, litter size was not altered appreciably. Therefore it would appear that the low conception rate was due to the small numbers of sperm used rather than to any detrimental effects on the sperm of the staining or sorting treatment.

The high incidence of perinatal death seen in our rabbits appeared to be linked with gestation length: Hafez (1970) described a relationship between a high perinatal death rate and prolonged

gestation in this species. The average length of gestation for rabbits bearing singletons was 35 days (26 observations) compared with 32.7 days (30 observations) for does carrying 6 or more offspring.

It might be expected that chromosomal damage produced by the staining or sorting process would be manifested by reduced fertility resulting from early embryonic death or in the production of deformed young (Lyon, 1988). The experiments described here showed that not only was there no evidence of any gross abnormalities in the offspring born in successive generations where the technique of inseminating stained or sorted sperm was used, but also that there was no deleterious effect on fertility attributable to this technique. The use of successive generations of rabbits obtained from stained sperm was an attempt to maximise the chance of obtaining congenital abnormalities in a situation where numbers of available progeny were strictly limited. The transitory drop in fertility we observed in the early generations of this experiment was shown to be due to inbreeding in our small colony.

These results do not necessarily contradict the findings of Libbus et al. (1987) that stained or sorted sperm were damaged. There were several differences in their sperm preparation technique which might cause some damage to the sperm. For example (1) mincing the cauda epididymides to extract sperm; (2) sonication of sperm; (3) chilling of sperm; and (4) staining with Hoechst 33342 at 9 μM (approximately 5 μg/ml). Sonication for longer than 15 sec has been reported to produce heat and could cause cell damage (Johnson et al., 1987a,b) while Martin et al. (1988) found that sonication and microinjection of sperm caused chromosomal abnormalities in up to 91% of sperm. In contrast to the technique reported by Libbus et al. (1987) we used ejaculated sperm which had not been subjected to treatments other than 'staining' or 'sorting'. We did not find it necessary to use such a high concentration of stain to achieve suitable levels of fluorescence for detection in the flow cytometer. Therefore, according to Smith et al. (1988), less chromosome damage would be expected in our sperm preparations. Libbus and colleagues (1987) concluded that stained sperm illuminated by UV laser light in the flow cytometer showed more chromosomal damage than stained but inactivated sperm. However it seems unlikely that much direct damage would result from UV at 351.1 and 363.8 nm since the maximum absorption for nucleic acids is at 260 nm.

According to Libbus et al. (1987) the microinjection of untreated hamster sperm into hamster oocytes resulted in damage in 20% of chromosome sets. However since there does not appear to be an adverse effect on fertility in naturally mated hamsters, it would seem likely that a selection process operates in vivo, removing embryos resulting from aberrant fertilizations. This mechanism may well be capable of coping with a small increase in chromosome damage caused by staining and sorting sperm.

In conclusion, it would appear from our results that inseminations with Hoechst 33342-stained or sorted sperm do not cause a measurable increase in the incidence of congenital abnormalities nor a dramatic fall in conception rate. The loss in fertility observed is almost certainly due to the small number of sperm used in some of these experiments. However it is not possible to state categorically that sperm did not suffer from chromosomal damage in our system since such sperm might be selectively inhibited in the female tract and so fail to compete with undamaged sperm. Alternatively embryos resulting from damaged sperm might fail to develop. Such in vivo selection is avoided in a situation where a sperm nucleus is microinjected into an oocyte and therefore we cannot compare our results directly with those of Libbus et al. (1987). It will be necessary to establish whether Hoechst 33342 staining and/or flow cytometry does cause damage to sperm DNA before any widespread use is made of sperm treated in this manner.

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